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ADVANCES IN ENZYMOLOGY
AND RELATED SUBJECTS

Volume II

CONTRIBUTORS TO VOLUME II

JULIUS BERGER, *Department of Botany, Connecticut College,
New London, Conn.*

MAX BERGMANN, *The Rockefeller Institute for Medical Research,
New York, N. Y.*

HENRIK DABE, *Dept. of Anatomy, School of Medicine and Dentistry,
University of Rochester, Rochester, N. Y.*

MAX DELBRÜCK, *Department of Physics, Vanderbilt University,
Nashville, Tenn.*

W. H. FULLER, *Agronomy Section, State College of Agriculture,
Ames, Iowa*

MARVIN J. JOHNSON, *Department of Biochemistry, University of
Wisconsin, Madison, Wis.*

A. G. NORMAN, *Department of Agronomy, State College of Agriculture,
Ames, Iowa*

J. J. PFIFFNER, *Research and Biological Laboratories, Parke, Davis
and Company, Detroit, Mich.*

E. A. HOUGHTON ROBERTS, *Indian Tea Association, Tocklai Experi-
mental Station, Cinnemara, P. O. Assam, British India*

HIROSHI TAMIYA, *Department of Botany, Tokyo Imperial University,
Hongo, Tokyo, Japan*

DONALD D. VAN SLYKE, *The Hospital of the Rockefeller Institute for
Medical Research, New York, N. Y.*

C. H. WERKMAN, *Bacteriology Section, State College of Agriculture,
Ames, Iowa*

E. J. WITZEMANN, *Department of Physiological Chemistry, University
of Wisconsin, Madison, Wis.*

H. G. WOOD, *Department of Bacteriology, State College of Agriculture,
Ames, Iowa*

E. ALBERT ZELLER, *Physiologisch-Chemisches Institut der Univer-
sität Basel, Switzerland*

ADVANCES IN ENZYMOLOGY
AND RELATED SUBJECTS

Edited by

F. F. NORD

*Fordham University
New York, N. Y.*

C. H. WERKMAN

*Iowa State College
Ames, Iowa*

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BACTERIAL VIRUSES (BACTERIOPHAGES)¹

By

MAX DELBRÜCK

Nashville, Tennessee

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¹ The present review does not attempt to present all lines of research concerned with the bacterial viruses. The following topics, which have been covered in recent reviews by other authors, have been omitted:

1. Bacteriophage therapy (Krueger and Scribner (8)).
2. Purification, concentration, and chemical studies (Northrop (10)).
3. Size determinations by ultrafiltration (Elford (6)).
4. Reversible and irreversible inactivation by heat and by chemicals (Krueger (5)).
5. Lysogenesis and its relation to the cancer problem (Andrewes (7)).

I. Bacteriophages as Viruses, General Properties, Present Problems

Twort in 1915 (79) and d'Herelle (43) independently in 1917 discovered and described an agent which destroys bacteria and which concurrently is reproduced. D'Herelle, who studied this agent extensively, called it "Bacteriophage," and considered it to be a submicroscopic living organism, which parasitizes bacteria. Bordet (14, 15) contended that the agent was a bacterial enzyme. He believed that the production of the agent within the bacterial cell is a function of the cell rather than a function of the agent itself. He thought that the introduction of the agent into the cell disturbs the natural metabolism of the cell in such a fashion that the cell starts producing material of the type introduced.

The difference of opinion expressed in the two hypotheses is one which concerns the complexity of organization and the biological autonomy of the bacteriophage. In d'Herelle's view the bacteriophages are small cells, in Bordet's view they are modified bacterial proteins. The issue is one which can only be settled by a clearer understanding of what actually goes on when the bacteriophage is reproduced. The experiments which have been devised in the attempt to settle this argument have not yet led to a clear understanding of the mechanism of phage reproduction.

The bacteriophages are to be classified with the animal and plant viruses as bacterial viruses. They share with the viruses which grow on the cells of higher plants and of animals the following properties:

1. They show no metabolism in the absence of suitable host cells.
2. They reproduce only if attached or inside a host cell. It seems that the host cell must be alive and must metabolize actively.
3. The range of sizes of the phages is the same as that of viruses (10-100 μ) (6).
4. They show host specificity. Some phages, for instance, will attack only one particular strain of one particular species, others have a wider host range, which, however, rarely transgresses the genus boundaries.
5. Chemically, they seem to consist of nucleoprotein (10).
6. The host-virus relation ranges from complete latency to complete destruction of the host.
7. The phages are not restricted to a part of the bacterial kingdom. Although there are many bacterial species, for which no phage has been

reported, this does not mean that such phages do not exist. The interest of the research workers was mostly concerned with the pathogenic species, for which almost invariably phages could be isolated. Suffice it to say that phages are known which are active against species of the following genera: *Bacterium*, *Bacillus*, *Micrococcus*, *Corynebacterium*, *Vibrio*, *Actinomyces*.

Interest in the bacterial viruses was at first sustained by the hope that they could be used as therapeutic agents against bacterial infections. During the first years after d'Herelle's original work, over six hundred papers on the subject were published. But success along these lines has been very meager (8). Phage is therapeutically inefficient, chiefly because it will rarely destroy all the cells of a given culture. A few cells remain which are resistant to the virus, and these form a secondary growth. The mechanism by which these resistant cells originate has not yet been cleared up.

The disappointment of the hope of using bacterial virus as a universal therapeutic agent against bacterial infections led to a temporary neglect of the subject. But around 1930 interest was revived by the recognition of the similarity of the phages with the animal and plant viruses. Andrewes (7) and Elford (6) established the diversity of the sizes of different phages, Schlesinger (71, 72) prepared rather pure and concentrated suspensions of a *B. coli* phage and studied it with dark field illumination and chemically. Burnet (4) studied the host specificity of a large group of phages and proved that the receptor spots on the bacterial cell, to which the phage is specifically adsorbed, are identical with a bacterial antigen.

Northrop and Krueger were led to take up the study of phage growth through their interest in enzyme production. They followed up their original quantitative study of the interrelation of the growth of the phage and of its host (45) by many studies of phage growth under varying conditions of bacterial growth. Krueger has claimed in recent years to have found evidence for the existence of a phage precursor in the bacterial cell (50-60).

At present two aspects of the phage problem seem to be of particular interest. One is the problem of the biochemical basis of the specific relation of the phage to its host. This problem is related to the antigen-antibody problem. A part of this problem is the study of the process of acquired resistance, which bears a relation to the unsolved problem of virus immunity in plants and in animals.

The other is the problem of phage growth. In the first place it is desired to know the precise relation of the phage growth with the biochemical functions of the host cell, and, secondly, one wants to know the nature

of the chemical process which secures the accurate reproduction of the virus itself. This has an obvious relation to the problem of the reproduction of the gene.

II. Distribution in Nature

In order to obtain a virus active against a given strain of bacteria one has to go to the natural habitat of the bacterial strain. For instance, virus against any *B. coli* strain will fairly regularly be found in filtrates of stool or sewage; virus against an intestinal pathogen will be found in the stool of patients who have recovered from such an infection. Similarly, with staphylococcal or streptococcal infections the virus can be isolated from the infected tissue. It is clear from these statements that virus of bacteria is of ubiquitous occurrence. Actually, it is even more widespread than these findings indicate. Let us consider for a moment the essential characteristics of a bacterial virus. These appear to be two: (1) the destruction of the host, and (2) the increase in amount of virus while attacking a host.

Of these two, the first characteristic is not a sufficient one, because there are many enzymes that will destroy bacteria without increasing concurrently in amount. One such enzyme is called lysozyme; it occurs in egg white and in tears. Another is called actinomycetene; it is a secretion of an actinomycete and has been studied extensively by Welsch (42). There are many others, many occurring in bacteria themselves. But the first characteristic, the destruction of the host, is also not a necessary one for a virus for, just as with plants and animals, there are bacteria which are *virus carriers*. Such strains harbor the virus and secrete it, but show no pathological symptoms. Such strains are called "lysogenic," because filtrates of cultures of such strains will always contain the virus, as can be shown by the use of some lysable strain as indicator. The virus will destroy the cells of the sensitive strain and concurrently grow on it. Of the two characteristics the second one, therefore, is the fundamental one, and the first one is only a symptom of the presence of virus which may or may not show up in any given strain. In order to detect a virus it is of course necessary to have at least one indicator strain that will show the symptom of lysis.

In lysogenic strains the secretion of the virus is analogous to the secretion of an extracellular enzyme. Northrop (67) has studied this analogy more closely with a strain of *B. megatherium*, which is lysogenic and produces the extracellular enzyme, gelatinase. He found that the two bacterial products are elaborated only by growing cells and roughly in proportional

amounts. In the opinion of the reviewer, this analogy is one which concerns the interrelation of the synthesis of virus and of enzyme, on the one hand, with the basic metabolism of the bacterium, on the other hand. There remains as a basic difference the fact that no virus is known which has enzymatic activity, *i. e.*, to act chemically on an extracellular substrate.

Lysogenesis was at first thought to be of rather infrequent occurrence. It was first reported by Lisbonne and Carrère in 1922 (64), and the relation of virus growth to bacterial growth was studied qualitatively by Gilde-meister and Herzberg in 1923 and 1924 (35a). In 1932 Burnet (19) reported a study of over one hundred stock strains of *Salmonella*, of which he found about half to be lysogenic, *i. e.*, carrying virus that was active against at least one of two indicator strains which he used in this experiment. . An even more remarkable case was reported by Bruce White in 1937 (82). He found that all Indian strains of *V. cholerae* were carriers of a certain virus *LL*, and all Chinese and Japanese strains of *V. cholerae* were not carriers and were sensitive to this virus. It follows that in all the work on cholera viruses that had been done with the Indian strains, every filtrate had contained this virus *LL*.

In lysogenic strains the symbiosis between the virus and the carrier is a very intimate one; it is a coordinated growth and the host cannot by any means be divested of its acquired physiological function (Burnet, 18). Every cell of the host strain is lysogenic, and in spore formers the symbiosis is carried through the spore stage (83). In this resistant phase of the host, the virus is resistant to all those treatments which leave the spore viable. Outside the bacterium the virus shows the normal heat instability of proteins. The increased heat stability of the virus within the carrier spore is not more and not less remarkable than the heat stability in the spore of the other protein components of the bacterial cell.

The phenomenon of lysogenesis has its analog in plant and animal viruses where it is called "indigenous virus." In these cases also the detection of the virus depends on the discovery of an "indicator strain." Thus tobacco plants are indicators for a virus which is carried by all American species of potatoes. In other cases the presence of a virus can only be inferred hypothetically on the basis of serological reactions. For instance, in the case of a tar sarcoma, which was transplantable by grafts, but not by tissue extracts, it could be shown that the new host developed antibodies against a known tumor virus, Rous No. 1 sarcoma. The presumption is that the tar sarcoma contained a virus which is serologically related to Rous' virus. Andrewes (7) has recently reviewed the possible relevance of these findings to the virus aspect of the cancer problem.

III. Methods of Assay, Survey

The simplest demonstration of the presence of a bacterial virus is by its dissolution of a liquid mass culture of some susceptible bacterial host. Two methods of assay are based on the use of this effect.

Serial Dilution Method.—One of these is an analog of the serial dilution method of bacterial assay and consists in the determination of the maximum dilution of the test sample, which will give lysis of a standard culture in a standard percentage of trials. This method is cumbersome and inaccurate, and is now seldom used.

Activity Method.—The second method was developed by Krueger (44). Here the *time* is measured which elapses between the introduction of a suitable sample of the virus suspension into a standard culture of the bacteria and the time at which lysis has attained a standard ~~stage~~. This method is rather accurate and simple in manipulation, and should give unambiguous results when used for the assay of virus which is not attached to or within bacteria. It has been used only by Krueger and Northrop and collaborators for their work on the growth mechanism of a staphylococcus virus.

Plaque Count Method (Figs. 3 and 4).—Lysis will in most cases also take place in mass cultures on solid media, and d'Herelle made use of this fact to develop a wonderfully simple and satisfactory method of assay, which is in effect an analog of the method of colony count for bacterial assays. One needs only to introduce a thick bacterial growth as the nutrient medium for the virus. The virus colonies will then show up as clear spots or "plaques," as d'Herelle called them, on the bacterial background.

The virtues and limitations of the methods of assay will be discussed after we have given an outline of the "Life Cycle" of bacterial viruses in sensitive hosts.

IV. The "Life Cycle" of Virus in Sensitive Hosts

A. ADSORPTION

The first step in the interaction between a virus and the bacterial host cell which is susceptible to lysis by this virus is the adsorption of the virus to the host cell. This adsorption is a specific one, *i. e.*, the virus will be adsorbed only by the sensitive hosts, and not by any other strain of bacteria, with the exception of lysogenic strains. For other exceptions see Rakićen (69).

1. Rates

It is easy to follow the progress of the adsorption reaction experimentally and to determine its rate.

After mixing a suspension with a known concentration of bacteria and of virus and allowing a certain time for adsorption, a sample of the mixture is diluted and centri-

fuged at a speed that will throw down the bacteria, and with them those virus particles that had been adsorbed up to the moment of dilution. The supernatant is assayed and gives the amount of unadsorbed virus. The adsorbed fraction is determined as the difference from the initial assay. It is found that the rate at which adsorption occurs is proportional both to the virus concentration and to the bacterial concentration, which means simply that it is determined by collisions between the two bodies. The absolute value of the rate is dependent on a number of factors, of which the physiological state of the bacteria and the electrolyte concentration are the most important.

Actively growing bacteria adsorb the virus much faster than do bacteria which are in the lag phase. For instance, with one *B. coli* strain the adsorption rate constant was found to be sixty times greater for actively growing bacteria than for bacteria in the lag period (26). Salt concentrations of about one per cent are optimal, but there are differences in the optimum for ions of different valency (Gratia (41), Krueger, *et al.* (47-49)).

2. Residual Fraction

If one follows the decrease in the concentration of unadsorbed virus in a mixture with bacteria in excess, one finds at first an exponential decrease which means that the adsorption is at any time proportional to the concentration of unattached virus. But, rather suddenly, the rate of adsorption slows down and a certain fraction of the virus, a few per cent, will not be adsorbed even in a long period of time. This free fraction could be due either to an adsorption-desorption equilibrium or to the fact that a certain fraction of the virus has a smaller affinity toward the host cell. Schlesinger (71) has investigated this problem in the case of a *B. coli* virus and has found that the residual virus is not due to desorption but has less affinity to the cell. The residual virus, however, does not differ in any essential from the main fraction, since its offspring behaves like the offspring of the normal type. Probably its affinity is reduced by the blocking action of some material which adhered to it, perhaps in the cell in which the virus was produced.

Krueger (46) has investigated the problem with his method of assay in the case of his staphylococcus virus, and has arrived at the opposite conclusion, *i. e.*, that the residual virus does not differ in affinity from the normal type, but is due to an equilibrium between adsorption and desorption. The reviewer (28) has reinvestigated Krueger's virus, using the method of plaque count, and has found results in conflict with those of Krueger, and in agreement with those of Schlesinger.

3. Adsorption Capacity

If one mixes bacteria with virus in excess one finds that a bacterium can adsorb a considerable number of virus particles. Eventually, however, saturation is reached and the point where this occurs gives an indication of the *adsorption capacity* of the bacterium. This capacity differs for different bacteria and different viruses and depends also on the physiological state of the bacteria. With strains of *B. coli* an adsorption capacity around 200 for actively growing bacteria and around 20 for starved bacteria was found (Delbrück, 27).

4. Irreversibility of Adsorption

Virus will also be adsorbed by bacteria which have been killed by heat or by radiation or by disinfectants. Attempts to split off the virus from the bacterium after adsorption without injuring the virus have not met with success, but have not been sufficiently extensive to lead to the conclusion that such a separation is impossible (42).

The nature of the specificity of the adsorption will be discussed in a later chapter.

B. LYSIS AND VIRUS LIBERATION

After a virus has been adsorbed by a metabolizing host cell, the course of events becomes for a short while unobservable. The cell appears normal and no virus is secreted by it. But the normality is only an apparent one; after a short while two striking events reveal profound changes: (1) the cell is lysed; (2) a large number of virus particles are liberated from it.

1. Lysis

The lysis of the cell can be observed in mass cultures by the clearing of the turbidity or it can be observed with individual cells under the microscope. As a matter of fact, what one sees under the microscope is very little. First the cell is there and appears normal, and the next moment the cell is gone, often without leaving any trace, and so fast that no intermediate stages can be seen. Bronfenbrenner, Muckenfuss and Hetler (16a), and Bayne-Jones and Sandholzer (13a), have published photomicrographic moving pictures of lysing cells. On these it can be seen that the cell sometimes swells up and sometimes fades out without change of shape. Delbrück (27) noted, with a strain of *B. coli*, that the lysis with swelling up

and the lysis without change of shape can be made to occur at will by adjusting the virus concentration.

Lysis from Without.—The first type occurs when the cell is surrounded by a very high concentration of virus. It is then attacked by several hundred virus particles simultaneously from without, and the cell is lysed in a few minutes, without giving rise to virus growth. All the adsorbed virus is lost. The cell begins to swell up at one end, gradually all parts swell, and the cell assumes an irregular shape and slowly fades out.

Lysis from Within.—If the cells are infected with only one or a few virus particles a longer time will elapse during which the cell shows no outward sign of anything abnormal going on. It may even continue to grow and divide, but this is a point for which only poor evidence can be advanced, since this latent time is about the same length as one division cycle. Then suddenly the cell fades out. In the case studied by Delbrück this happened in about two minutes, and a faint outline of the rod remained visible for a long time afterwards. In other cases the fading-out goes much faster, often in fractions of a second. In this case one has the impression that a membrane ruptures, and that the fading-out is due to a diffusion of the cell content into the surrounding medium. In the case of lysis from without, the cell wall is apparently attacked and is slowly digested away.

2. *Virus Liberation*

The liberation of virus from the cell, like the lysis of the cell, is not a gradual, but a sudden process. It takes place after an interval of time from the moment of infection which is equal to that time which elapses between infection and lysis. Since both of these times vary somewhat from one bacterium to another, and both cannot be determined for the same individual bacterium, the equality of these two time intervals is difficult to prove conclusively. D'Herelle was the first to propose that the two processes, lysis and liberation of the virus, occur simultaneously. Recent accurate experiments have supported this view.

Let us consider in greater detail the evidence concerning the liberation of the virus. The method of assay with plaques is well suited to bring out this feature, because it will register an infected bacterium up to the moment of burst as one plaque, and immediately afterwards, when the virus content is dispersed into solution, as many plaques. In a mass culture of bacteria, in which a large number have been infected with virus at a certain time, the plaque count will therefore stay constant as long as no bacteria start liberating virus. As soon as this happens a rapid rise in the plaque count takes

place, until all the originally infected bacteria have liberated their share. The plaque count will then have risen by a factor equal to the average yield of virus per infected bacterium (burst size).

By following the plaque titer of a growing mixture of bacteria and virus, with assays at intervals of one or two minutes, these three quantities can be determined, *viz.*, the *constant period*, the *rise period* and the *burst size*. Figure 1 gives an example of a "one step growth curve."

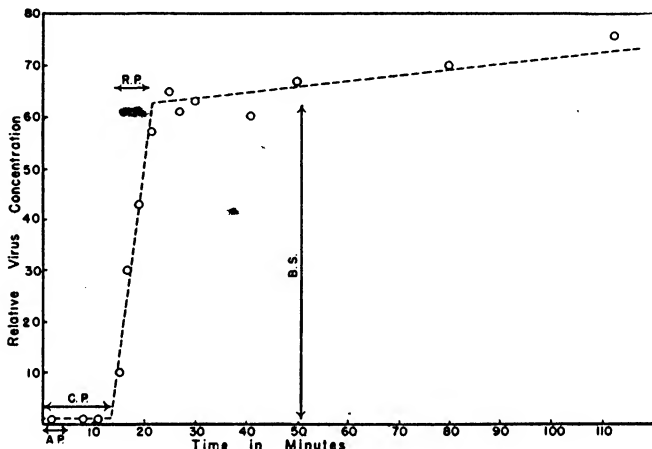


Fig. 1.—One step growth curve of a *B. coli* virus (P_3 on B_3). A.P. = Adsorption period (5 min., 45% adsorbed during this period). C.P. = Constant period (13 min.). R.P. = Rise period (5 min.). B.S. = Burst size (62, corrected for adsorption: $62/0.45 = 138$). The adsorption mixture contained 5×10^7 bacteria/cc. and 3×10^7 virus particles/cc. At the end of the adsorption period this mixture was diluted 1:200,000 in broth.

The essential feature of such an experiment is the proper control of the adsorption rate. In the beginning one wants the bacteria, as nearly as possible, infected simultaneously, so as to start them all off at the same time. Therefore, one mixes virus with a rather high concentration of bacteria. Under the best conditions, most of the virus particles will then be taken up in two or three minutes.

As soon as the first bacteria start liberating new virus, these additional viruses may infect more bacteria. This leads to further virus growth, possibly before all the originally infected bacteria have liberated their share. Thus one obtains a growth curve with several more or less pro-

nounced steps (Fig. 2). To prevent such reinfection, one must dilute the original mixture of bacteria and virus *after* adsorption has taken place, and *before* virus liberation begins. If one dilutes by a sufficiently high factor one will not get reinfection for a long time; the virus titer will stay constant, and the burst size can be determined with accuracy.

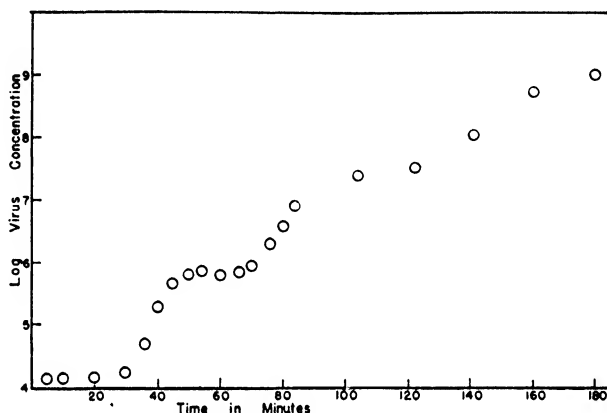


Fig. 2.—Growth curve of a *B. coli* virus with several steps (P_1 on B_1).

One step growth experiments have been carried out only on a few strains of *B. coli* virus. These will be referred to as follows:

B_1 and P_1 (Ellis and Delbrück, 1939).

B_2 and P_2 (Delbrück, 1940).

B_3 and two viruses, P_3 and P_4 (Delbrück and Luria, 1941).

A few experiments of this sort have also been performed with Krueger's staphylococcus virus. These strains will be called: B_s and P_s .

Constant Period.—Determinations of the constant period can be done with an accuracy of about one minute. Under controlled conditions the values obtained from different experiments do not fluctuate outside this limit of the experimental accuracy.

Dependence on virus: With growing bacteria of strain B_3 it was found (29) that with one virus (P_3) the constant period is 13 minutes, with another virus (P_4) on the same host it is 21 minutes. The division time of the bacteria under these conditions coincides with neither of these values; it is 19 minutes. (Microscopic observations showed that in these cases the first lyses appeared after about 13 and 21 minutes, respectively.)

Dependence on temperature: With strains B_1 and P_1 Ellis and Delbrück (30) found the following dependence of the constant period on temperature:

Temperature, C.	Constant period, min.	Division period of the bacteria, min.
37	30	21
25	60	42
16.6	180	About 120

There is a constant ratio $3/2$ between the constant period of virus growth and the division time (growth rate) of the bacteria. The coincidence suggests a connection between bacterial assimilation and the processes which lead to virus liberation.

Dependence on physiological state of the bacteria: With strains of B_1 and P_1 Delbrück (27) measured the constant period of virus growth both with bacteria in the rapid division phase and with bacteria that were taken into fresh broth from a culture that had been aerated for 24 hours. Such bacteria are very small and will respire and grow in size, but they will not divide for about 90 minutes after transfer to fresh broth. He obtained the following results:

Bacteria	Constant period of virus growth
Dividing every 30 minutes	17 minutes
Old, not dividing for 90 minutes	30 minutes

A similar lengthening of the constant period was obtained with B_2 and P_2 (29) and with Krueger's virus (28). It is clear from these results that there exists no simple relation between the division cycle and the constant period.

Independence on multiplicity of infection: An important and peculiar finding is the fact that the constant period is not measurably altered when the bacteria are infected by an excess of virus, as long as the excess is not so great as to cause lysis from without. This was first reported by Ellis and Delbrück (30) with up to four-fold infection. It has recently been verified with viruses P_3 and P_4 on bacterium B_3 with up to fifteen-fold infection (29). The length of the constant period under these conditions was not altered by more than one minute. It would appear, from this finding, that the process which leads to virus liberation, although it is

initiated by the virus infection, is uninfluenced by the number of viruses that infect the cell.

Rise Period (Variability of Latent Periods).—Under the conditions of one step growth, the plaque titer will rise until all the bacteria that were infected during the short initial adsorption period have dispersed their share of newly formed virus into solution. The duration of this rise period is an indication of the variability of the latent periods of virus growth. The beginning of the rise gives the shortest latent period and the end of the rise gives the longest latent period. This variability of the latent periods is in most cases considerable, comparable in length to the minimum latent period. For instance, with the strains B_2 and P_2 whose constant period was 17 minutes, Delbrück (27) found a rise period of 16 minutes. In this case, therefore, the latent periods of the bacteria ranged between 17 and 33 minutes. A part of this variability is only apparent, because in these experiments¹ the adsorption period was five minutes; the bacteria were therefore infected over this length of time and the true spread of the latent periods may therefore have been shorter. This point was tested recently (29) with another strain of bacteria and virus, where the constant period was 13 minutes. By shortening the adsorption period to one minute, the length of the rise period could here be reduced to five minutes.

The constant period gives the minimum latent period, and this, as explained before, is a well-defined and reproducible parameter. The end of the rise period is not as clearly defined. On the one hand, it is more difficult to determine experimentally, because the accuracy with which the plaque counting method determines virus titers is limited by a constant per cent error. In the beginning of the rise the plaque titer may rise by a factor 20 in one minute, whereas at the end of the rise it will increase by a factor of only 1.4 in one minute. It is obvious that the latter is more difficult to determine. Nevertheless, it seems indicated by the experiments that for some viruses the rise does end as sharply as it starts, *i. e.*, that practically all bacteria yield their share within sharply defined time limits, and not according to some statistical distribution similar to an error curve. In other cases the rise may go up to about 80 per cent of its final value in a few minutes, and then continue to rise for 30 minutes before it reaches the maximum value. In such a case we have to assume that about 20 per cent of the bacteria are for some reason delayed in their liberation of virus.

Burst Size (Average).—The factor by which the virus titer increases in a one step growth experiment is determined by the average yield of virus per bacterium. In actual experiments a correction has to be ap-

plied to take account of the viruses that were not adsorbed at the close of the initial adsorption period and which therefore did not have a chance to infect a bacterium.

Determinations of burst sizes give values between 100 and 200 if the bacteria are in the rapidly dividing phase. With bacteria from 24 aerated cultures very much smaller values are obtained, around 20. The bacteria in this phase are also much smaller (27, 29). Delbrück pointed out a possibly significant equality between the burst size and the adsorption capacity of the bacteria. Since the adsorption capacity is probably determined by the number of specific receptor spots on the bacterium, this equality means that the bacterium can produce as many virus particles as it contains receptor spots for the virus. With B_1 and P_1 Ellis and Delbrück (30) obtained a burst size of about 60. In these experiments the bacteria were taken from a one-day not aerated broth culture, and were therefore in a phase intermediate between that of rapid division and extreme starvation. That the burst size is a rather constant characteristic of the bacterium in a given state was indicated by the finding that it is independent of the temperature, between 16.6° and 37° C. (30). It was also found with B_1 and P_1 that the burst size is not affected by multiple infection (30). In recent experiments (29) with B_3 and P_3 and P_4 an increase in the burst size by perhaps a factor two was found with up to fifteen-fold infections. For these strains the adsorption capacity has not yet been determined.

Burst Size (Individual).—In the one step growth experiments the *average* burst size of a large number of bacteria is determined. Burnet devised a technique with which it is possible to determine the yield of virus of *single* bacteria. A large number of viruses are mixed with an excess of bacteria. After allowing time for adsorption, and before the beginning of the first burst, this mixture is diluted until the suspension contains about one infected bacterium per 0.1 cc. The suspension is then distributed into many small vials, 0.1 cc. or less into each. These samples are incubated until all bursts have occurred, and then plated each on a separate Petri dish. These plates will show either no plaque, if the sample did not contain a virus, or many plaques, if the sample contained an infected bacterium which liberated its yield during the incubation of the sample. The number of samples which will contain none, one or two infected bacteria will be determined by Poisson's distribution law. In order to have only few samples with more than one infected bacterium, one has to make the average number of infected bacteria per sample smaller than one. Then most samples will contain no infected bacterium. In order then to arrive at a statistical distribution for the burst sizes, one has to handle a very large number

of samples. The technical difficulties involved in this have not yet been overcome, but fairly large numbers of bursts were determined by Ellis and Delbrück (30) for B_1 and P_1 and by Delbrück (27) for B_2 and P_2 . The unexpected result of these experiments was that the variability of the burst size is extremely large, the burst sizes ranging between very few, and over 200 virus particles, without any pronounced maximum in the size distribution curve. This variability of the individual burst sizes is far too great to be accounted for by the variations in the sizes of the bacteria. If further experiments should verify these findings and show that the variability is of general occurrence, it would mean that the latent period of virus growth within a bacterium is not terminated by the attainment of a definite number of viruses within the bacterium, but by some other process which is initiated by the infection, and which runs a course which is more regular than that of the virus growth, and which therefore leads to lysis after a fairly well-defined time interval.

C. DISCUSSION OF METHODS OF ASSAY

The experiments described in the previous sections verify and amplify d'Herelle's picture of the "Life Cycle" of viruses in sensitive hosts. The virus attaches itself to the bacterium. Certain receptor spots on the bacterium are responsible for this specific attachment. There may be up to 200 receptor spots on one bacterium to which a given virus may attach itself. After the attachment the virus multiplies upon or within the bacterium for a certain period, but no virus is released from the bacterium during this period. Suddenly the bacterium is lysed and a large number, up to 200, of viruses are released.

The *plaque counting method* of assay determines the number of "infective centers" which are present at a certain moment in a suspension, by spreading a suitably diluted sample of the suspension on a nutrient agar plate, together with a large number of sensitive bacteria. The bacteria grow and give a thick continuous sheet of bacteria. Any virus particle will grow concurrently at the expense of the neighboring bacteria and will eat a hole in the bacterial sheet—a plaque. Similarly, an infected bacterium, which may contain a considerable number of virus particles, will eat a hole in the bacterial sheet at the place where it comes to lie on the plate when the sample is spread. A plaque is therefore caused either by one free virus or by one infected bacterium.

The number of plaques, which appear after incubation, is never quite

equal to the number of infective centers actually present, for two reasons. First, some virus particles may fail to attach themselves to a bacterium before the bacterial sheet has grown so thick that the bacteria cease to grow, and therefore cease to be a suitable substrate for virus growth. Second, some virus particles may attach themselves to dead bacteria, of which there is always a non-negligible fraction, even in actively growing cultures. For these two reasons, the efficiency of plating is never 100 per cent; under best conditions it is around 80 per cent. The efficiency of plating is not quite the same for free virus particles and for infected bacteria, because in the latter group no loss occurs on account of failure of adsorption. The efficiency of plating varies with the conditions of plating, but is very constant if the condition of the plates and of the plating bacteria is well controlled. The plaque number is strictly proportional to the concentration of the virus suspension (30), and fluctuations between duplicate plates correspond to the expected sampling errors.

Krueger's activity method (44) of assay determines the number of virus particles in a given suspension by adding a suitably diluted sample to a standard suspension of sensitive bacteria, and incubating the mixture under standard conditions. Both virus and bacteria grow, but the virus grows much faster and eventually so far exceeds the bacteria in number that the number of bacteria rapidly decreases by lysis and the turbidity of the suspension decreases. The time at which a certain standard degree of clearing is reached, gives a measure of the number of virus particles originally introduced, and by reference to one of the other methods of assay this measure can be converted into number of virus particles.

Those who have used this method report that it is convenient in manipulation and gives accurately reproducible results (error of single determinations about 5 per cent).

A special consideration is needed when it is desired to compare assays of "extracellular" and "intracellular" virus, as determined by this method. In the first place, a single virus particle will give a different assay value, depending on whether it is in the free state or adsorbed to a bacterium. The reason is that the free virus particle will spend a considerable time before it meets a bacterium to which it can attach itself and grow, and will therefore be delayed in its contribution to the eventual lysis of the test suspension. Similarly, a virus particle, which is adsorbed to a cell in the lag period, will assay lower than a virus particle which is adsorbed to a rapidly dividing cell, because, in the former case, the virus growth within the bacterium will have a longer latent period and a smaller eventual yield of virus than in the latter case.

We (28) have determined the constant periods of virus growth and the yields for Krueger's virus with the following results:

Bacteria	Constant period	Yield per bacterium
Dividing phase	30 min.	60
Lag phase	50 min.	15

The difference in the assay value between free and adsorbed virus, and between virus adsorbed to dividing and not dividing bacteria, must be very considerable. They can be estimated from Krueger's data on the adsorption rates and from our data on the respective latent periods and burst sizes. Such estimates give a factor of about five for the ratio of the assay value of an adsorbed virus as compared to a free virus, and a similar factor for the ratio of the assay value for virus adsorbed to dividing and to not dividing bacteria. These corrections have not been taken into consideration by those who have used the method. In the opinion of the reviewer, many of the interpretations of experiments in which this method has been used are, therefore, erroneous. For instance, in the experiments in which the concentration of extracellular and intracellular virus was compared by this method (66), the calculated concentration ratio may be in error by a factor five. More serious is the error incurred in the interpretation of a series of experiments by Krueger and his collaborators (50-60), in which it is claimed that proof has been found for the existence of a virus precursor in "activated bacteria," *i. e.*, bacteria in the dividing phase. In these experiments the virus is mixed with "activated bacteria" in the cold for a few minutes, and then titrated in the usual manner. The concentration of the "activated bacteria," with which the virus was mixed, was so high that the virus would be adsorbed in a few minutes. These virus particles will, therefore, assay higher than free virus particles or virus particles adsorbed to bacteria in the lag phase, and will thus simulate a true increase in virus. In the opinion of the reviewer, these experiments serve to illustrate the ambiguities of the particular method of assay employed, and do not indicate the presence within the cell of a virus precursor, which can be transformed into virus in a resting cell in the cold.

These objections to the activity method of assay do not apply to those experiments in which only assay values of free virus particles are compared, as in experiments on the inactivation of virus by heat or by chemicals (5). They apply to some extent to the experiments on adsorption of virus by the sensitive host (46), because, as we have seen, a given suspension of virus is inhomogeneous with respect to its affinity to the bacteria. The virus particles which remain unattached after a certain time in a mixture of virus with bacteria are, therefore, a selected sample, selected for slow adsorption rate, and will assay lower than the main fraction of virus particles, if assayed by the activity method. Estimates of the residual fraction of virus will therefore tend to be too low.

V. The Specificity of the Host-Virus Relation, Compared with the Serological Specificity of the Host and of the Virus

It has been stated above that the relation between the virus and its host cell is a specific one. The nature of this specificity has been explored in

considerable detail. It resides in certain receptor areas on the bacterium, to which the virus can attach itself. The affinity of the receptor spot to the virus is of a similar nature to that between an antigen and its antibody, and it has been studied in connection with serological work. In fact, it has been shown that the receptor spots on the bacterium are identical with one or the other of the heat-stable polysaccharid antigens of the bacterium. Paralleling the antigenic complexity of most bacteria, there exists also a diversity of serologically distinct viruses that may attack the same bacterium. The subject has been reviewed thoroughly by Burnet (9). We will give only a brief survey of the results. For convenience we will use the following notations:

B—Bacterium.

b—Receptor spot on bacterium (a bacterial antigen).

β —An antibody against the antigen *b*.

P—A virus with affinity for the bacterium.

π —An antibody against the virus *P*.

1. $B + \pi \longrightarrow$ *no reaction*

The virus is always serologically distinct from the host cell. Antiserum which is prepared by injecting lysates into rabbits will contain antibodies of two kinds, those against the bacterium and those against the virus. The former are absorbable by the bacteria, the latter are not.

The serological character of a virus is independent of the host on which it is grown. This important fact was first shown by Gratia in 1922 (36) with a staphylococcus virus which could be grown either on an aureus strain or on an albus strain. It has been verified by many other workers. Burnet (9) states that "the serological nature is one of the most definite intrinsic characteristics of a bacteriophage, and comparative serological work provides the best basis for a classification of any large group of bacteriophages." (The constancy of the serological type of a virus strain stands in contrast with the comparative variability of the virus in its affinity to the host. By cultural selection methods, the affinity of a virus to a certain host can be greatly modified.)

All viruses which attack a certain group of bacterial species can be classified into a few groups, which show serological cross reactions within the group, and no cross reaction between groups. The viruses of one group are of similar sizes. Such classifications were made for the coli-dysentery group by Burnet (20a), for the *V. cholerae* by Asheshov (13), and for staphylococci by Burnet (23).

Sertic and Boulgakov (76) have shown that one arrives at the same classification of the viruses by applying the following criterion: Viruses *A* and *B*

belong to the same group if *A* does not lyse the resistant secondary growth of a bacterial strain which arises after lysis under the influence of *B*, and *vice versa*. This finding of Sertic and Boulgakov supports the view that some of the surface elements of the virus which are antigenic are identical with those which unite with the bacterium, and that the resistant type of secondary growth represents a bacterial variant, which has lost the corresponding receptor spot.

2. $BP + \pi \longrightarrow agglutination$

The adsorption reaction between the bacterium and the virus has been described in a previous section. It has there also been stated that the adsorption capacity of a bacterium is high, a single bacterium being able to adsorb up to 200 virus particles.

The fact that the bacterium becomes coated with virus particles if it is exposed to an excess of them, can also be shown by the fact that such virus-coated bacteria can be agglutinated by serum which only contains antibodies against the virus (20).

3. $b + P \longrightarrow bP$

The receptor spots on the bacteria can be isolated from bacterial extracts or from filtrates of old bacterial cultures (Levine and Frisch (63), Burnet (21, 22), Bruce White (81), Ellis and Spizizen (32)). Such preparations act as virus inactivators. The polysaccharid combines with the virus and blocks the part of the virus which has an affinity to the bacterium.

The kinetics of this reaction has been studied by Ellis and Spizizen (32). They found the remarkable result that the rate of virus inactivation at 0° C. is proportional to the square root of the concentration of the inhibitor, as if the inhibitor existed mainly in the form of a dimer, and only the monomer was active as inhibitor. The dimerization must be easily reversible, because at 37° the reaction was found in simple proportion to the concentration of the inhibitor.

Sometimes the receptor substance is also responsible for a macroscopic colony character of the strain. For instance, Bruce White (81) described a virus which will attack only S-forms of *V. cholerae*. All rough strains of *V. cholerae* are resistant to this virus. Polysaccharid isolated from the smooth strains will inactivate this virus, while similarly prepared polysaccharid from the rough strains will not do it.

The identity of the receptor spots *b* with the polysaccharid antigens of the bacterium can be inferred from the fact that susceptibility to a certain group of serologically similar viruses is always correlated with the presence

of one definite antigen in the host. We refer to Burnet's monograph (9) for examples.

The identity is proved more directly by showing that the *b* does indeed react with bacterial antiserum, *i. e.*, by showing that the "virus-inactivator *b*" is in turn inactivated by β (Burnet (21), Rakietsen, Rakietsen and Doff (68)). Tiffany and Rakietsen (78) showed that *b* can also be blocked by antiserum in the intact bacterium. Such bacteria will not adsorb the virus.



The reaction between a virus and its antibody can be studied by three methods, *viz.*, by the inactivation of the virus, by agglutination of the virus (Burnet (19a), Schlesinger (71)), and by agglutination of virus coated bacteria (Burnet (20)).

The kinetics of the inactivation reaction has been studied extensively by Andrewes and Elford (11, 12) and by Burnet and Lush (9), without, however, leading to completely unambiguous results with regard to the details of this process. Andrewes and Elford (11) showed that the per cent inactivation is independent of the virus concentration. This means that the virus particles are inactivated individually, not by their aggregation. They also found that even at the highest serum dilutions at which they obtained inactivation, the antibodies are in excess of any attainable virus concentration. The serum could never be depleted of antibody by the addition of virus.

More difficult to answer is the question whether a virus particle can be inactivated by its combination with a single antibody particle. The problem can be attacked by following the time course of the reaction. If a single antibody is sufficient to inactivate a virus particle, the fraction of surviving virus particles should at first decrease exponentially, without an initial lag period. However, the experimental curves with diluted serum do seem to indicate the presence of a short initial lag period. There is also indirect evidence that the inactivation reaction at least in some cases can proceed in several steps. The surviving fraction appears to be somewhat altered, in that it gives smaller plaques and a longer latent period, and is less filterable.

The rate of virus inactivation slows down after the main fraction has been inactivated, just as the rate of adsorption of virus to bacteria slows down after the main fraction has been adsorbed. However, the virus particles which are resistant to inactivation by serum are not the same as those with reduced affinity toward the bacteria, as was shown by Andrewes and Elford in two ways (11). On the one hand, it was found that

the "adsorption survivors" showed normal inactivation by serum, and, on the other hand, the "serum survivors" showed normal affinity toward the bacteria.

These findings can be explained by the following hypothesis (25). If an antibody combines with the virus, it may or may not block the point of the virus which can combine with the bacterium. If it does block this point the virus will be inactivated. If it does not block this point, it may yet block a neighboring point. Such a virus particle is active toward the bacterium, though with delay, since it cannot properly grow until it has been freed of the attached antibody. Finally, the antibody may combine in such a fashion that it does not block the attachment point to the bacterium, but does block the point of attachment of another antibody which could block this point. The first antibody would thus protect the virus particle from inactivation by other antibodies. Such a mechanism could account for the origin of the serum-resistant fraction, which gives smaller plaques.

VI. Virus Mutations

There are two instances of mutations of bacterial viruses recorded in the literature with sufficient detail to justify their inclusion in this review.

Gratia (39) described a virus which is normally carried on a lysogenic strain of *B. megatherium*. Plated with a sensitive strain of *B. megatherium*, it gives, as a rule, turbid plaques, with a lysogenic secondary growth. However, in a very small fraction of cases one finds also clear plaques. If the virus from such a clear plaque is isolated, one finds that it "breeds true." This mutant will also lyse the original lysogenic host strain.

Burnet and Lush (24) described a similar case with some additional experiments. These authors were working with a virus *C*, which was active against a *Staph. albus* strain *SF*. Again this virus was one which produced ample secondary growth, so that the plaques looked turbid, and the secondary growth was lysogenic. The plaques had another peculiar characteristic—they grew indefinitely, with the secondary growth simultaneously spreading out in the inside, so that the plaque had the appearance of a gradually widening ring. As in Gratia's case, the mutant *C'* of this virus was one of greater "virulence," giving very little secondary growth and consequently clear plaques. What secondary growth occurred was not lysogenic. The two viruses were found to be serologically identical.

The two secondary bacterial cultures, which arise by the action of virus *C* or of its mutant *C'*, respectively, we will call *SF/C* and *SF/C'*. They are, of course, resistant to the action of the virus which originates them. But in this case they are also resistant to the other virus (*i. e.*, *SF/C* is

resistant to C' , and SF/C' is resistant to C). Burnet and Lush made use of this fact in studying the origin of the secondary culture of C , *i. e.*, of SF/C . A plate on which are spread a large number of bacteria SF and of virus C' will, after incubation, show only very few colonies, but, if prior to the addition of virus C' , some virus C is added to the bacteria, some of the bacteria will be transformed from SF into SF/C , which is resistant to C' . The infection of the culture SF with the weak virus C will, therefore, protect it from the action of the virulent mutant C' . Infection with C will, therefore, result in a rich secondary growth resistant to C' . From experiments, in which C' is added a few minutes after C , it is seen that in these few minutes a large number of bacteria had become resistant to the action of C' .

It follows that virus C attacks the strain SF in two alternative ways. Either it lyses the attacked cell (in about 80 per cent of the cases) or it becomes a latent virus of the cell, simultaneously protecting it from the action of the virulent mutant.

The virus C' can be obtained as a rare mutant from aging cultures of the lysogenic strain SF/C , just as the virulent mutant was obtained, in the case described by Gratia, from the strain which was lysogenic with respect to the avirulent form.

Clearly, these findings open up an experimental approach to a wide range of problems. Mutations of the kind described probably occur quite often. Analysis of the conditions which control their appearance would be of value to the general mutation problem. The work of Burnet and Lush seems to show that they offer also a powerful tool for the analysis of the induction of a type of virus immunity which depends upon the introduction of an indigenous virus.

VII. "Lysines"

Under this heading may be grouped a number of observations which seem to show that in the process of lysis by viruses one or several enzymes, besides the virus particles, are involved.

Gratia and Rhodes (37) noted that heat-killed staphylococci were lysed by homologous virus, but the virus was not the active agent in this process. It was found (38) that live cells of the same strain were a better source of the agent. If these were added in very small amounts to a thick suspension of dead bacteria the suspension cleared almost completely. During this process the live bacteria grew a little at the expense of the disintegrating dead cells. If a few live bacteria are plated on an agar plate impregnated with a thick suspension of dead bacteria, the bacterial colonies developing from the live cells will develop a "halo," *i. e.*, a zone of lysis of the dead cells around them. In this case, therefore, the live bacteria secrete an

enzyme which is capable of attacking the dead cells of the same strain (and of other strains).

Sertic (74, 75) studied a phenomenon which at first sight has only a superficial similarity with Gratia's observations. Also many viruses develop around the plaque which they form on solid medium a sort of "halo," *i. e.*, a zone which is not clear and not as turbid as the background layer of bacteria. With some viruses these "halos" are very striking; they may be much bigger than the plaque proper, and may continue to widen long after the plaque has ceased to grow (Fig. 3, plate on lower left side). Sertic showed that these "halos" are caused by an enzyme, which diffuses from the plaque, and does not kill the bacteria, but apparently digests certain capsular materials of the bacteria. This enzyme acts also on bacteria which have been killed by chloroform. The digestion of the capsular material changes the appearance of the bacterial sheet from thick and glistening to thin and transparent.

In subsequent papers (75, 77), Sertic has attempted to prove that this enzyme is a product of the virus and not an intracellular enzyme of the bacterium which is set free by the lysis of the bacterium. In the first place the enzyme is not found in culture filtrates of the bacteria or in extracts of the bacteria. It is also not liberated, if the same bacterium is lysed by another virus, which produces plaques without "halos." Gratia (40) has confirmed these findings of Sertic, and has proposed the names "lysino-genic" for those viruses which cause the secretion of this enzyme from the bacteria which they lyse, and "lysinosensitive" for those bacterial strains which elaborate the capsular material that can be digested by the lysine. He found that a non-lysino-genic virus acting on a non-lysinosensitive strain may induce a secondary growth which is lysinosensitive, *i. e.*, which elaborates the capsular material. He also found that the enzyme may be liberated by the lysis of hosts which do not elaborate the capsular material. In later publications, Sertic and Boulgakov (76a) studied the serological characteristics of the lysines and of the capsular materials of a number of strains.

The lysines of Sertic and of Gratia do not really lyse the bacterial cell, but digest some capsular material. Schuurman (73) described a lysine originating from a bacterium which is lysed by a virus, and which does lyse other bacteria. He selected for his study a bacterial strain which is an obligatory aerobe. By proper control of the oxygen supply he was able to show that the virus would not grow when the bacterial metabolism was checked by lack of oxygen, but the enzyme would continue to act on the bacteria, even when these were killed (by heat or by chloroform), and at low temperatures (6° C.).

Evans (33-35) has described a phenomenon which she observed with viruses attacking strains of streptococci, and which she ascribed to the activity of "nascent virus." She tested three viruses against a large number of streptococcus strains and found that the range of activity of each virus was greatly enhanced if a small amount of a sensitive strain was added to the suspension of the strain to be tested. In other words, during the growth of the virus on the sensitive strain, an agent was liberated which helped the activity of this virus against otherwise resistant strains of streptococci. This agent was apparently quite unstable, since it disappeared after filtration of a lysate of the sensitive strain.

In the opinion of the reviewer, the phenomena described in this section are probably manifestations of the autolytic enzymes of the bacterial cells, which are liberated when the host cell is lysed by the virus.

VIII. Inactivation by Ionizing Radiations

Early studies of the effect of X-radiation on bacterial viruses had shown that rather high doses are required to produce an appreciable effect on the activity of viruses (around 50,000 r units) (84). Quite recently, Wollman, Holweck and Luria (86), and Luria and Exner (65) have published detailed studies of this effect, which contain results of the utmost importance, both for the virus problem and for radiation biology in general.

It was found that there exist radiation effects of two kinds: First, an *indirect* one, in which the virus is inactivated by some decomposition product of the water. This effect is predominant if the virus is suspended in distilled water or in a salt solution. If a trace of foreign protein is added (10^{-4} gm./cc. or more) this indirect effect disappears, apparently because the foreign protein competes with the virus for the active decomposition product of the water, and thus protects the virus from the harmful effects of this product. Secondly, there remains a residual effect of the radiation on the virus which must be ascribed to ionizations which occur in the virus itself, *i. e.*, a *direct effect* of the radiation.

1. Single Hit, Homogeneity of Size of Virus Particles

The direct effect can be shown to be due to single ionizations. The fraction of active virus particles which remains after irradiation with a certain dose of x-rays decreases exponentially with increasing dose. This exponential decrease was followed down to very small fractions of residual virus (2×10^{-7}). There was no indication of the presence of a more resistant fraction of viruses, such as would be expected if the virus popula-

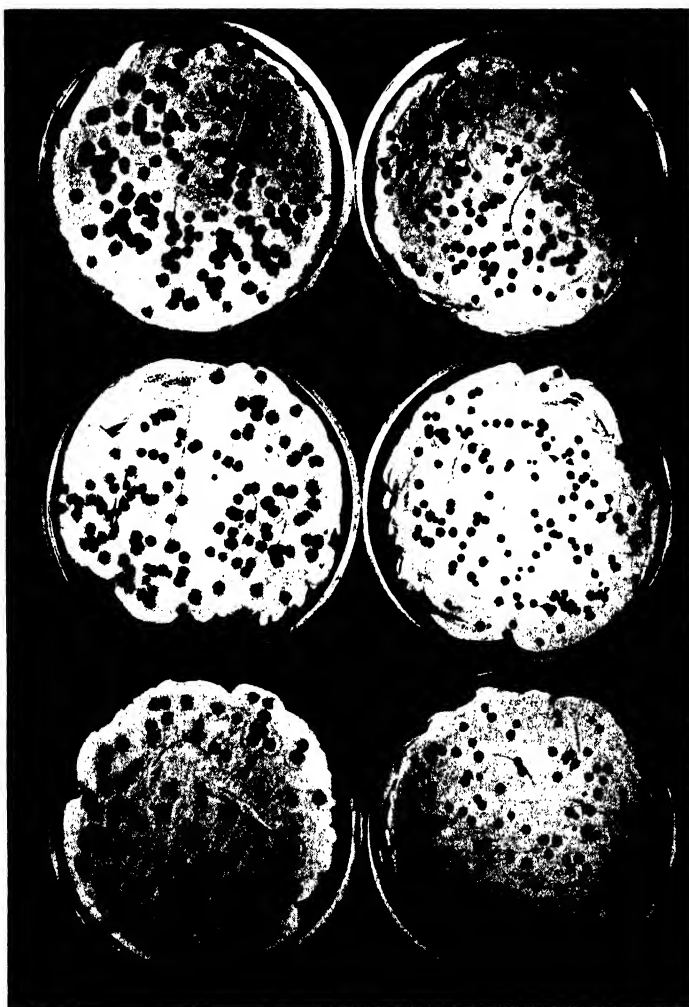


Fig. 3.—Plates from an experiment on the inactivation of virus by x-rays. Duplicate plates for three doses (0, 30,000 and 50,000 r) showing decrease in the titer of active virus. The lower left plate was incubated 24 hours longer in order to show the development of halos around the plaques.

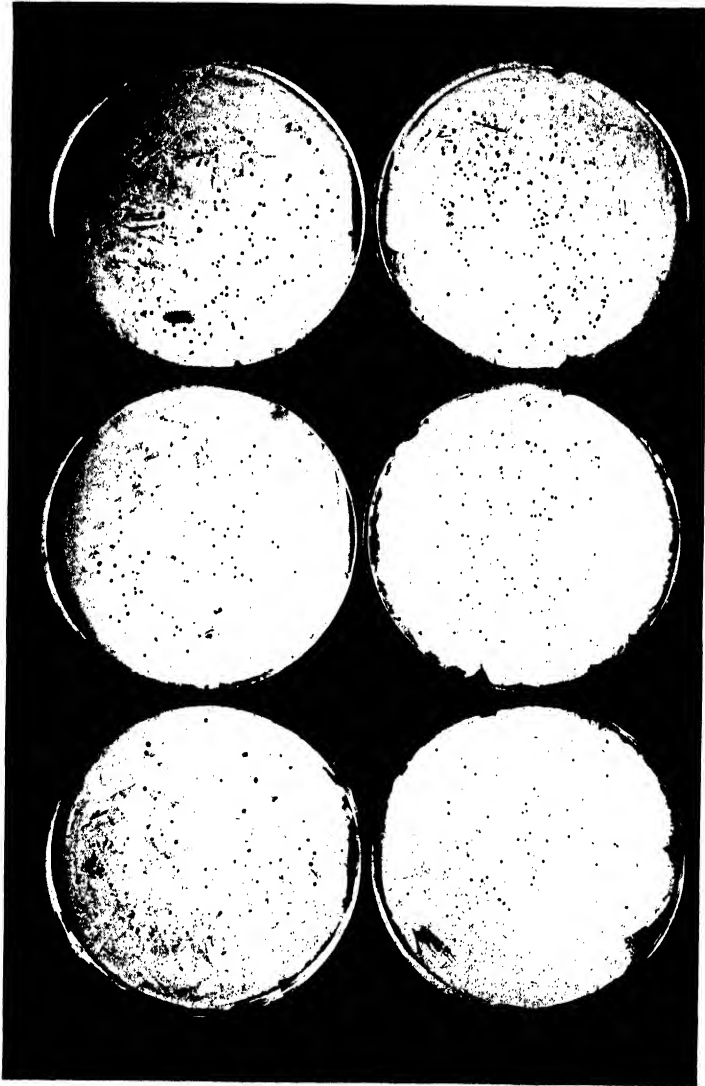


Fig. 4.—Same as Fig. 3, with a different virus (Krueger's staphylococcus virus 3K). (Courtesy of S. E. Luria and F. M. Exner.)

tion had any inhomogeneity of size. On the contrary, at very high doses the survivor fraction fell off a little more steeply than in the beginning. *It follows that the essential part of the self-reproducing entity is identical in all individuals of a given population of a pure strain of virus.*

2. Sensitive Volume and Particle Size

From the slope of the dose-survivor curve a so-called sensitive volume can be calculated; it is the volume which receives on the average one ion cluster at the dose which reduces the number of active virus particles by a factor $1/e$. It is often supposed that this volume is approximately equal to the true volume of the particle. But such an interpretation is in most cases very uncertain, because one does not know whether ionizations which occur outside of the particle may cause its inactivation by an indirect mechanism. On the other hand, perhaps a large fraction of the ionizations which occur within the particle may not inactivate it. For these reasons, sensitive volumes are often regarded as fictitious quantities and of doubtful physical significance.

However, in the case of the bacterial viruses (and of tobacco mosaic virus, Lea (62)), these ambiguities are eliminated. The results concerning the indirect effect, which we cited above, show that outside hits can indeed inactivate the virus, but they also show that this effect can be suppressed by the addition of protective foreign protein. The residual effect must therefore be due to direct hits. Calculation now shows that the sensitive volume is equal to the physical volume (as determined by filtration experiments). It follows that very nearly every hit within the physical volume actually does inactivate the particle. In other words, we have here a unique case, where we can be reasonably certain that we are dealing with the simplest type of a "hit-theory," *since the effect is produced by single hits, and indeed by every direct hit and only by these.*

We consider this analysis of the radiation effects as convincing evidence regarding the true size of the self-reproducing entity which is the virus.

IX. Attempts to Obtain Growth of Virus without Growth of the Host

Krueger and Northrop (45) in their first comprehensive study of the interrelation of bacterial growth and of virus growth, had reached the conclusion that virus growth is dependent on bacterial growth. In later work they modified this view to some extent. Krueger and Fong (52) claimed to have obtained virus growth without bacterial growth by adjusting the pH of the medium to 6 and the temperature to 28° C. With *B. megatherium* Northrop (67) obtained the same result at a pH of 5.5. These

findings were the starting point for Krueger's experiments which attempted to show the existence of a precursor of the virus in growing bacteria (50). While we are not convinced of the existence of a precursor for reasons which have been stated in Section IV, 3, the possibility of obtaining virus growth without bacterial growth is plausible on theoretical grounds, and is also supported by an experiment of Ellis and Spizizen (31). These authors found a slight growth of the virus if the bacteria were suspended in a solution of glycine in distilled water (200 mg. per cent). The bacteria did not grow in this medium. Whether they metabolized the glycine was not determined directly, but it can be inferred, since without the glycine no virus growth occurred. Although none of these experiments is very convincing, the last-mentioned one opens an important line of attack. It should be possible to determine, by standard biochemical methods, which part of the basic bacterial metabolism is necessary for the growth of the virus.

X. Conclusion

In conclusion it may not be amiss to point out some of the items of information which we lack, and without which we cannot hope to come to a deeper understanding of the nature of a virus, and also, perhaps more important, to point out a paradox which is embodied in the information which we believe to have, and which bars us from a comprehensive understanding of the host-virus relation.

We know that the growth of the virus and the lysis of the host are not necessarily correlated phenomena. In the lysogenic strains, the virus growth occurs without lysis. This lack of correlation is exhibited even more strikingly in the relation between animal and plant viruses and their host cells. Here the effect of virus growth on the host cell varies widely from virus to virus, and from host to host, and from one type of host tissue to another. We conclude, therefore, that the lysis in the case of a sensitive bacterial strain is an indirect effect of the virus growth. The growth of the virus will always dislocate to some extent the harmonic equilibrium of the metabolism of the host cell. In the case of a sensitive host it dislocates the equilibrium beyond a critical limit, so that the cell will be destroyed. The destruction is of the peculiar type of a sudden lysis after a rather short time. One has the impression that the immediate cause of the lysis lies in the rupture of the semipermeable protoplasmic membrane of the cell, but it is not possible to trace the cause of this rupture further back. The time at which lysis occurs is not determined by the virus growth directly, but through some intermediate cause. One would like to know, then, whether at the time of lysis the virus growth has run

to completion, or is still going on at a rapid pace. In other words, what determines the yield of virus to be obtained by a given host cell? Do we obtain, say, 200 virus particles from a host cell because the cell did not provide raw material for more virus particles, or because the cell happened to have synthesized just 200 virus particles by the time the cell was lysed and the enzymes were dispersed? We believe that the first alternative is the more probable one, because multiple infection does not increase the yield, and because change in temperature, which changes the latent period, does not affect the yield.

It is at present not possible to formulate a working hypothesis concerning the rôle of the receptor spots for the *growth* of the virus. In some respects they seem to be similar to the virus, in others they seem to be antithetic. They are similar, inasmuch as the bacterium produces them (in the absence of virus) just as it produces virus, if the virus is introduced into the system. The experiments of Alloway (10a), concerning the type specific pneumococcus polysaccharides point to an even closer analogy. He showed that pneumococcus organisms of any smooth type contain a "transmissible agent" which is capable of evoking the production of the type specific polysaccharide in a rough pneumococcus strain of any origin. This is somewhat analogous to a bacterium becoming lysogenic with respect to a virus which may be introduced from outside. One might be led to think that the receptor spots themselves represent part of a virus whose growth is in harmonic equilibrium with the bacterial growth, and that the virus which we introduce from the outside displaces the indigenous one, and that it can do so by virtue of its similarity with the indigenous one. There are strong indications that similar viruses do interfere with each other when they enter the same host cell. This picture explains why there exists such a definite correlation between specific adsorption of a virus and the possibility of growth of the virus in the cell which adsorbs it.

On the other hand, there are serious objections to the view that the receptor spot is similar in structure and in function to the virus itself. For one thing, the receptor is supposed to be made up of polysaccharide, whereas the virus is supposed to consist chiefly of nucleoprotein. Secondly, the relation between virus and receptor spot, as we have seen, is similar to the relation between antigen and antibody, and such a relation is not one of chemical similarity, but of complementariness. The receptor spot and the virus fit together like die and coin, their union is due to steric fit and to electrostatic attractions.

Stated briefly, the paradox is this: if the antigen serves its function

to adsorb the virus, the antigen seems unnecessary for the growth of the virus (contrary to experience). On the other hand, if the antigen is similar to the virus in structure and the virus synthesis replaces the antigen synthesis, the antigen seems unsuited to adsorb the virus (contrary to experience).

It is of importance that the dependence of the virus growth on the metabolism of the host cell should be examined more closely. One often hears the view expressed, particularly among scientists who work with animal and plant viruses, that the cell provides a suitable nutrient medium for the virus and that the virus grows in this medium as a bacterium would grow in a cell-free nutrient medium. But the indications are that the dependence of the virus on the cell goes deeper. It seems that in the synthesis of new virus not only storage products of the cell are used as building blocks, but also short-lived intermediate products of metabolism are employed. The virus makes use of the metabolic machinery of the cell for its own needs. The oxidation-reduction cycle and the phosphorylation cycle of some cell metabolite may be directly involved. Such a study will require the analysis of the growth of host and of virus in the presence of a variety of substrates and inhibitors, under aerobic and under anaerobic conditions. In the opinion of the reviewer, the problem of autocatalytic synthesis in the cell may be approached in this manner with promise of success. It is likely that its solution will turn out to be simple, and essentially the same for all viruses as well as genes (29a). The bacterial viruses should serve well to find this solution, because their growth can be studied with ease quantitatively and under controlled conditions. The study of the bacterial viruses may thus prove the key to basic problems of biology.

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THE KINETICS OF HYDROLYTIC ENZYMES AND THEIR BEARING ON METHODS FOR MEASURING ENZYME ACTIVITY

By

DONALD D. VAN SLYKE

New York, N. Y.

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I. Deviation of Enzyme Reactions from the Monomolecular Curve

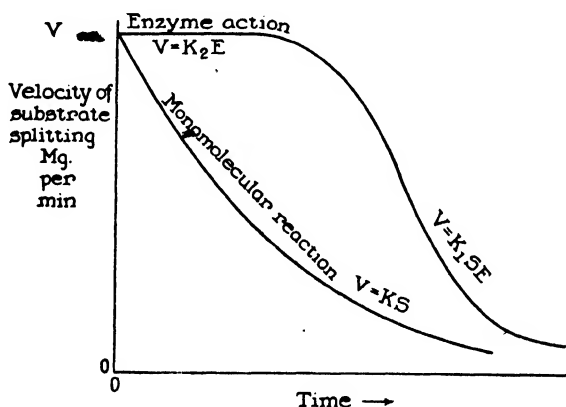
The "monomolecular" mass law is merely a statement that a constant fraction of a reacting substance decomposes or otherwise reacts in each time unit. Consequently the absolute amount of product formed per minute is proportional to the amount of mother substance present. The reaction follows the lower curve of Fig. 1. As the reaction progresses the amount of mother substance still present diminishes and the rate of the reaction consequently falls as indicated by the concave curve, both the residue of substance and the rate ultimately approaching zero. This character has given the curve of such a reaction also the name of "die-away curve." It is shown by many phenomena other than chemical decompositions. For example, if gas escapes from a leak in a pressure tank, the rate of escape slows down as the pressure in the tank falls, and the

removal of the gas follows the die-away curve. This name, therefore, has an advantage over "monomolecular reaction curve" in that the name, "die-away curve," carries no assumption as to the nature of the process, whether it is physical or chemical.

The equation of the die-away curve is

$$-\frac{dS}{dt} = V = KS$$

where S is the active concentration of the reacting substance, V is the velocity of the reaction, and t is the duration time of the reaction. The relation between reaction time t , and the fraction, $(a - S)/a$, of initial



Effect of duration of reaction on velocity, as substrate concentration falls.

Fig. 1.—Time curve of simple monomolecular decomposition reaction contrasted with typical curve of substrate decomposition by an enzyme.

substance that still remains at this time, is found by integrating the velocity equation, and is the familiar "monomolecular" exponential equation,

$$\frac{S}{a} = e^{-kt} \text{ or } t = \frac{1}{K} \log \frac{a}{S},$$

where a is the initial substrate concentration at the start of the reaction.

When the inversion of cane sugar is catalyzed by acid the hydrogen ions merely speed up the process of decomposition; the inversion continues to follow the die-away curve. The only effect of the acid is to increase the fraction of cane sugar that is inverted per minute.

When, however, instead of acid, the catalyst is the enzyme, invertase, the die-away curve is followed only when the sucrose solutions are dilute.

If the inversion is started in a 10 or 20 per cent sucrose solution, a very different type of curve is encountered. Until most of the sucrose has been inverted the rate of formation of invert sugar continues constant; it does not slow down, and during this phase of the reaction there is no suggestion of a die-away (horizontal part of upper curve of Fig. 1). This is the first phase of the reaction. Eventually, however, when sucrose concentration has been lowered to 3 or 4 per cent, the rate curve does begin to fall, and finally it falls parallel with the concentration of the remaining sucrose, and finishes as a die-away curve. This is the second phase of the reaction; it imitates a monomolecular reaction. But it is only an imitation. The two-phase character of the entire reaction shows that it is not a simple monomolecular decomposition.

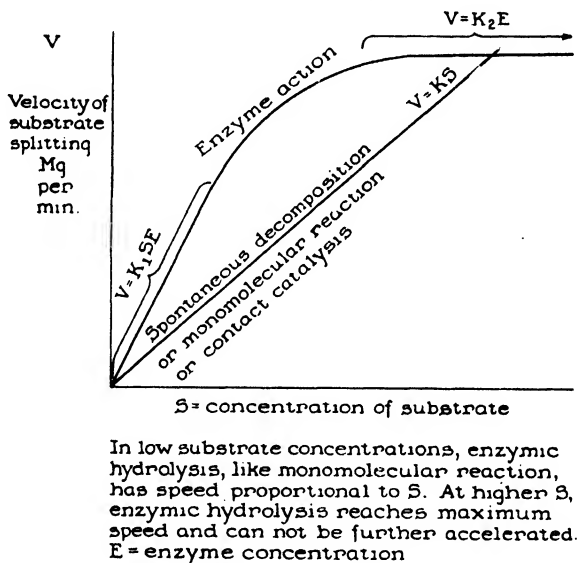


Fig. 2.—Effects of substrate concentration (S) on velocities of spontaneous decomposition and of decomposition by enzymes.

The above cited facts concerning invertase were revealed in 1902 by Henri (8) and by Adrian Brown (3), and were confirmed by Michaelis and Menten (13) with careful control of the pH . Other hydrolytic enzymes were shown to show the same two-phase type of reaction. H. T. Brown and Glendinning (4) demonstrated it for diastase, Michaelis and Abderhalden (12) for a peptidase, Van Slyke and Cullen (14) for urease. And it has since been met in various enzymatic reactions (16). Archibald (1)

in our laboratory has recently demonstrated it for arginase. This last work cannot be mentioned without referring to the researches of Andrew Hunter and his colleagues, Dauphinee and Pettigrew (9, 10), whose studies of arginase have developed a beautiful analytical method for arginine, and with the work of Hellerman (7) have furnished the foundation for investigations of this enzyme's kinetics.

II. Initial Reaction Velocity

The effect of substrate concentration on rate of reaction can be studied more accurately by starting the reaction with varying substrate concen-

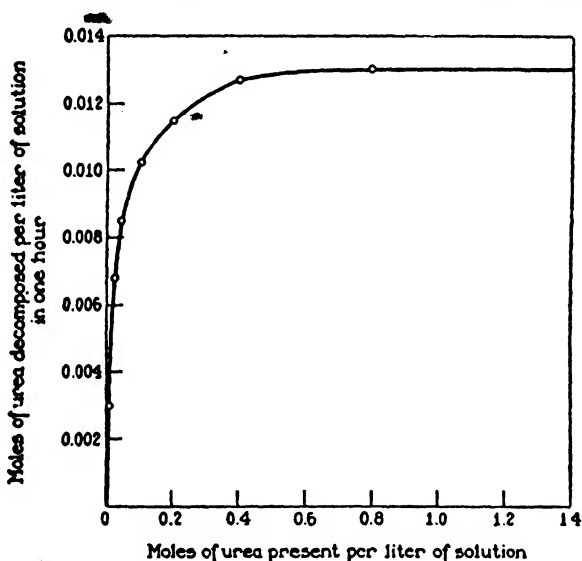


Fig. 3.—Effect of urea concentration on velocity of urea hydrolysis by urease.

trations, and measuring the initial reaction velocities, rather than by following one reaction curve all the way down to its finish. When initial reaction speeds are used the enzyme is not embarrassed by the accumulated end-products. One then obtains curves of the type shown in Fig. 2. Brown (3), Henri (8), and Michaelis and Menten (13) early made such studies of inversion with varying initial sucrose concentrations. So to speak, they followed the inversion curve backwards from dilute to concentrated substrate solutions.

As the initial sucrose concentration was increased from a fraction of a per cent upwards the amount of sucrose inverted per minute showed a parallel rise (part of curve following equation $V = K_1SE$ (Fig. 2)) until the concentration was increased to somewhat over 1 per cent. When sugar concentration reached about 4 per cent the inversion velocity reached its maximum. Further increase (part of curve following equation $V = K_1E$ (Fig. 2)) of sucrose up to 30 per cent could not push the reaction any faster. Results illustrating similar behavior of urease (14) and arginase (1) are shown in Figs. 3 and 4.

The characteristics of the two-phase curve and the deductions from them have been critically and exhaustively reviewed by Michaelis (13), by Lineweaver and Burk (11), and especially last year by Wilson (16). I shall attempt no such complete review, but only a brief discussion of the possible mechanism of the two-phase reaction, and of certain applications of the facts to the control and measurement of enzyme activity.

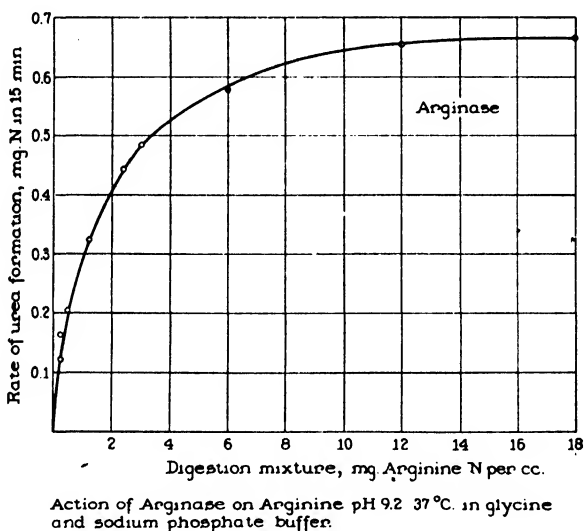


Fig. 4.—Effect of arginine concentration on velocity of arginine hydrolysis by arginase.

III. Mechanism of the Two-Phase Reaction

Nearly all authors who have encountered the two-phase reaction of enzymes have concluded from it that the enzymes combine with their substrates; that it is while thus combined that the substrates are hydrolyzed; that in low substrate concentration only part of the enzyme is combined at any one moment; and that this incomplete saturation of the enzyme in dilute substrate solutions is the cause for the failure of the enzyme to show its maximum activity in such solutions.

Concerning the mechanism of the reaction between enzyme and substrate the different authors reached similar views in different ways. The following is the explanation suggested by Cullen and Van Slyke (14), which seems to accord with the facts as well as any hypothesis, and to involve as few assumptions.

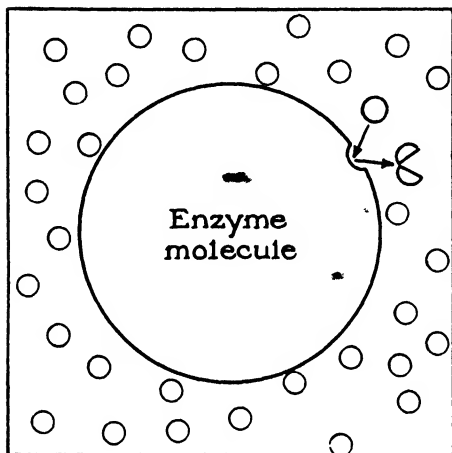


Fig. 5.—Diagram illustrating assumed mode of hydrolysis by an enzyme. The small circles represent substrate molecules.

It is assumed that each substrate molecule, before it is decomposed, first combines with the enzyme. Later, after a definite time interval, the substrate is split and thrown off as its products. In the case of urease these products are ammonia and CO_2 , in the case of invertase they are glucose and fructose, etc. After the products are ejected the place of combination on the enzyme molecule is left vacant until another substrate molecule makes contact (Fig. 5). Then the combination and decomposition are repeated. The time required for a single cycle is the

sum of the time required for another substrate molecule to hit the enzyme on the combining point, plus the time the enzyme then takes to split the substrate molecule and eject its products. *The more abundant the substrate molecules are about the enzyme, the shorter will be the probable path of the next substrate molecule to the combining point on the enzyme, and hence the shorter will be the average time interval during which the enzyme is left uncombined, and therefore inactive.* If the substrate concentration is great enough, this inactive interval becomes negligible compared with the interval required for decomposition. *When substrate concentrations are at or above this level, the enzyme works at full speed, because its unused intervals are negligible, and further increase of substrate concentration cannot push the reaction rate any faster.*

It is possible that both phases of the reaction may be reversible (the arrows in Fig. 5 would then be reversible). In such a case reversion of the hydrolytic process may occur, and the enzyme act to produce syn-

thesis instead of hydrolysis. In the cases of some enzymes such reversion is in fact attainable under properly chosen conditions. In the particular enzymes that we have discussed, however, reversion appears to be slight, and the hydrolysis tends to go to a finish.

IV. Quantitative Formulation of the Kinetics of the Two-Phase Reaction

The above explanation of the course of the reaction was formulated (14) as follows:

The time required for the cycle can be expressed as the sum of the intervals required for the two consecutive phases.

$$\text{Time required for one cycle} = \frac{1}{K_c S} + \frac{1}{K_D} \quad (1)$$

$$\begin{aligned} S &= \text{concentration of substrate.} \\ K_c &= \text{velocity constant for combination of enzyme and substrate.} \\ K_D &= \text{velocity constant for decomposition of combined substrate.} \\ -\frac{dS}{dt} &= \text{velocity of substrate hydrolysis, or} \\ -\frac{dS}{dt} &= \frac{K}{\text{Time required for 1 cycle}} \text{ or} \\ -\frac{dS}{dt} &= \frac{1}{\frac{1}{K_c S} + \frac{1}{K_D}} \quad (2) \end{aligned}$$

When concentration of S is large enough so that $1/K_c S$ becomes negligible in comparison with $1/K_D$ (i. e., when the combination of enzyme and substrate is practically instantaneous, in comparison with the decomposition phase) the above velocity equation simplifies to

$$-\frac{dS}{dt} = K_D \quad (3)$$

Hence K_D can be determined very simply as the hydrolysis rate in sufficiently concentrated substrate solutions to give maximal reaction speed.

Integration of equation (2) gives, as the time curve of the reaction:

$$t = \frac{1}{K_c} \log \frac{a}{S} + \frac{a-S}{K_D}, \text{ or } t = \frac{1}{K_c} \log \frac{a}{a-x} + \frac{x}{K_D} \quad (4)$$

where a , S , and t are as on p. 34, and x is the concentration of reaction product formed. When x is small compared with a , as at the start of a reaction with large a , the reaction may approximate the linear, $t = x/K_D$. As x increases the curve may approach the "monomolecular,"

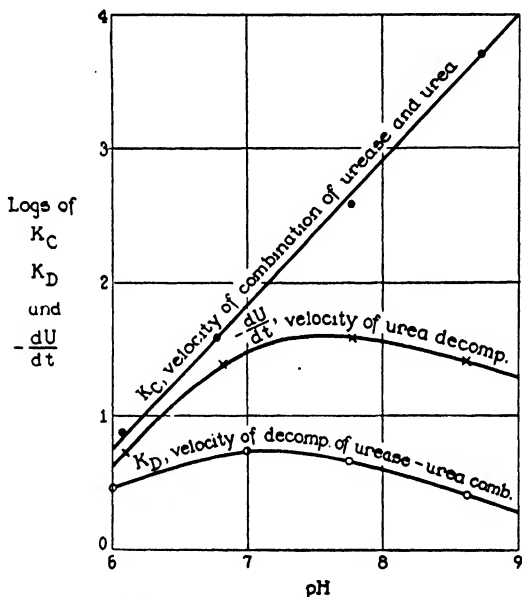
$$t = \frac{1}{K_c} \log \frac{a}{a-x}$$

If a , or K_c/K_D , is small, the monomolecular curve may be simulated from the start.

And, as we have seen (Fig. 1) this curve may in fact be simulated when the substrate concentration falls to a low level.

The above equations have been found to fit the two-phase curve of enzyme reactions when disturbing factors, such as change of pH during the reaction (14), and other effects of the products, are avoided.

Michaelis and Menten (13), as a measure of the substrate concentration required to half saturate an enzyme, introduced a constant, which is the substrate concentration that will cause the enzyme to work at half its



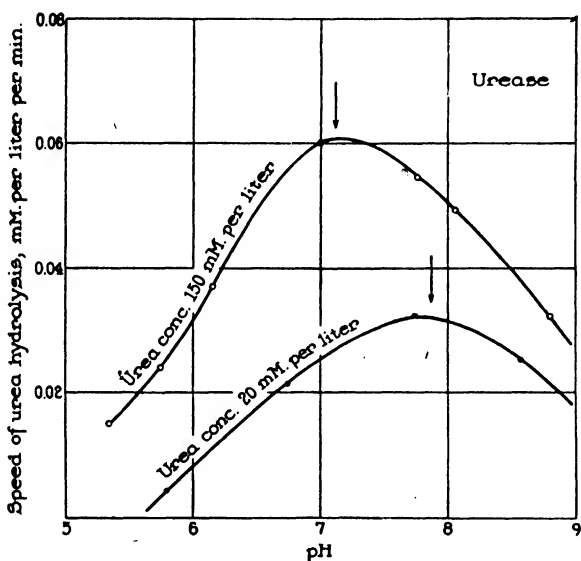
Effects of pH on the two phases of urease action.
Urea conc., $\underline{U} = 0.15 M$

Fig. 6.

maximum rate. It is obvious from equation (2) that these conditions are met when $K_c S = K_D$; hence that with variation in K_c , such as may be caused by pH change, the value of S for the Michaelis constant will vary inversely as K_c . The Michaelis constant is a most useful value, but it may be well to point out that it can be called a constant only for defined conditions; for example a drop of 1 unit in pH will increase the Michaelis constant for urease 11-fold (14).

V. Use of the Two-Phase Curve in Practical Control of Enzyme Action

One object of enzyme study is to learn how to control the action in order to use enzymes efficiently. Such, in fact, was the object with which Cullen and Van Slyke undertook the study of urease; they needed to use the enzyme for urea determinations. To be able to predict the action of such an enzyme from start to finish it is necessary to know its behavior in each of the two phases. One can use high concentrations of substrate to measure what we have called the decomposition velocity constant, and study the effects of pH and other factors on this phase of the action. The results



Shift of optimum pH to alkaline side by increasing substrate concentration.
Phenomenon due to different effects of pH on K_1 and K_2 .

Fig. 7.

will indicate the most effective conditions for the action of the enzyme in the more concentrated substrate solutions, such as are likely to be present at the beginning of the action before much of the substrate has been destroyed. Then one can make similar studies of the effects of pH, temperature, etc., with dilute solutions, in the range where the reaction follows the die-away curve. The results in this case will indicate the best conditions to use to bring the action to a finish, to decompose the last traces of substrate as its concentration approaches zero.

In the case of urease, variations in pH showed extraordinarily different effects on the speeds of the two phases of the action.

It is seen from Fig. 6 that K_c , the combining velocity, rises with pH , and the effect is so great that over the pH range 6 to 9 each unit of pH increase multiplies the combining velocity eleven-fold. Since it is the combining velocity that governs the rate of reaction in high dilutions, it follows that, to bring the decomposition of urea to completion, it is desirable to *finish* the digestion in alkaline solution, where K_c is greatest.

On the other hand, the decomposition constant, K_D , which governs the rate of hydrolysis in more concentrated solutions of urea, has its optimum pH at 6.8 so that it is desirable to *start* the reaction near this pH .

The difference in effects of pH on the two phases of urease action makes the optimum pH for the action of the enzyme dependent on the concentration of urea in which the urease works. In Fig. 7 are curves for two urea solutions that are only moderately far apart in concentration. However, it is obvious that the optimum pH is more alkaline in the more dilute solution.

To make the enzyme complete the decomposition of urea in minimum time, when it begins with a urea solution of more than tenth molar concentration, it is therefore desirable to start the action at about neutral pH , and let it turn alkaline as the digestion proceeds and the urea concentration decreases. This can be arranged by using enough phosphate buffer to keep the solution near neutrality during the early stages, but not enough buffer to prevent the development of alkalinity by the ammonium carbonate formed as the hydrolysis reaches its later stages. These conditions were used by Van Slyke and Cullen (14) in developing a rapid analytical method for urea determination with the enzyme.

The effects of pH on the two phases of the action of arginase have been studied by Archibald (1) and are shown in Fig. 8.

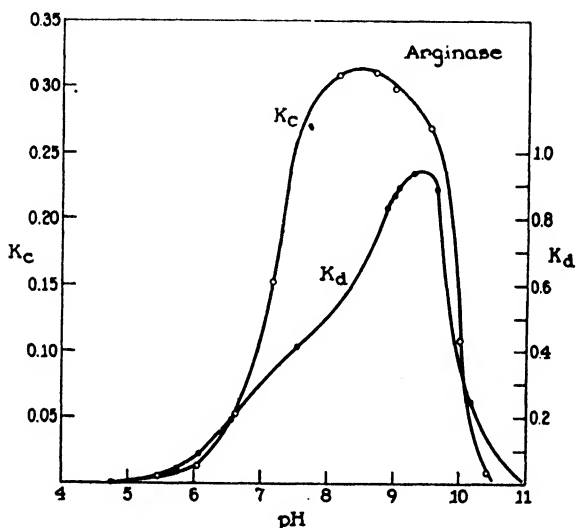
It is seen that the relative effects of pH on the two phases of arginase are altogether different from those noted in urease. Each enzyme must be studied for its own peculiarities in the two phases of its action.

The foregoing discussion has been limited to the action of hydrolytic enzymes. However, it appears that the same sort of kinetics is shown by at least some other types of enzymes. Two interesting examples have recently been described. Cori and Cori (6) have found a case in which the action of the enzyme is not hydrolysis but condensation, the formation of glycogen from glucose phosphate, under the influence of *phosphorylase* from mammalian tissues. The Michaelis constant was found to be 4.7 molar glucose-1-phosphate, indicating that formation of glycogen attains half its maximal rate when the substrate reaches this concentration. An

altogether different type of enzyme is the *transaminase*, which transfers ammonia back and forth between glutamic acid and alpha-keto acids. This enzyme has recently been found by Cohen (5) to show the typical kinetics of the two-phase reaction.

VI. Measurement of Enzyme Activity

We will discuss three techniques for measurement of enzyme activity, and endeavor to show how each of them fits into the picture of the two-phase reaction.



Arginase. Effects of pH on K_c and K_a

Fig. 8.

1. Activity Measurement in Substrate Concentrations High Enough to Give Maximal Rates

The simplest way to measure the activity of an enzyme is to use so high a concentration of substrate that the enzyme works at its top speed. The initial speed of the reaction is measured. The amount of substrate split in a given time, such as 15 minutes, is directly proportional to the enzyme concentration. The action is permitted to run long enough to permit an exact measurement of the amount of substrate decomposed. But it is not run so long that the substrate concentration is lowered into the range

where the enzyme works at only part of its maximal speed. A short reaction period, such as 5 to 30 minutes, may be desirable. The short period has several advantages: (1) It limits the amount of substrate decomposed, so that the concentration remains high enough to support maximal activity of the enzyme. (2) The short reaction prevents the accumulation of such amounts of end-products as might retard the action of the enzyme. (3) It minimizes the chance that a significant part of the enzyme may be inactivated during the reaction period. In the case of urease Archibald has found a period of five minutes advantageous (1),

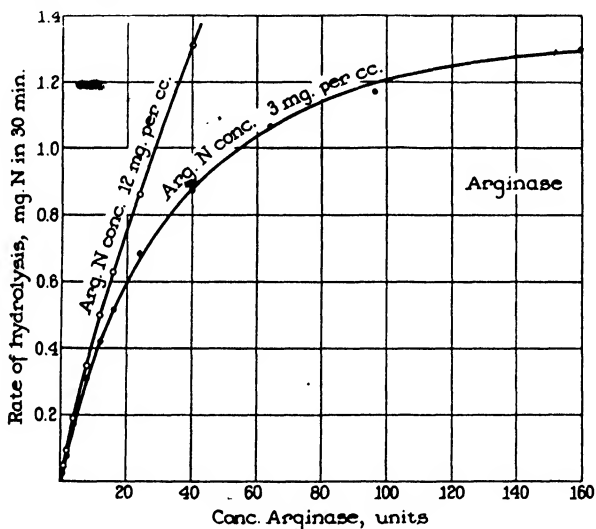


Fig. 9.—Curves showing that amount of arginine decomposed in 30 minutes approximates a linear function of the arginase concentration (up to 40 units) when the initial arginine concentration is 0.214 molar (12 mg. of arginine N per cc.), but not when the initial arginine concentration is one-fourth as great.

with the reaction taking place in the chamber of the manometric gas apparatus (15). At the end of the five minutes the action is stopped by admission of acid, and the CO_2 of the ammonium carbonate that has been formed is measured.

Even with short reaction periods, it may be necessary to take precautions against loss of enzyme activity during the reaction. Two examples may serve as illustrations. When urease activity is measured in the manometric apparatus (15), it is necessary to use only a milligram or two

of the crude jack bean urease prepared by acetone precipitation. Larger amounts would produce too much CO_2 to measure in the apparatus. But the dilute solutions of urease are unstable. A 10 per cent solution of the crude urease, consisting mostly of accompanying water-soluble jack bean proteins, is stable; it shows little loss of activity in several days. But a solution containing only a milligram per cc. may lose a measurable amount of activity in a few minutes. It has been found that egg albumin acts as well as the natural jack bean albumin to protect the urease against loss of activity. Consequently, for activity measurements, the urease is dissolved in a solution of egg albumin, and its reaction with urea is carried out also in the presence of albumin. Arginase likewise has been found by Archibald to lose activity rapidly when Hunter's glycerol liver extract, which itself is a stable preparation of the enzyme, is highly diluted with water. In the case of arginase, addition of albumin fails to protect. However, addition of a manganous salt greatly retards the loss of activity, and in part the apparent activation of arginase by manganese, which has been thoroughly studied by Hellerman (7), seems to be due to this protective effect.

2. Activity Measurement in Substrate Solutions So Dilute That the Monomolecular Law Is Simulated

Instead of using substrates in such high concentrations that the enzyme shows its full activity, one may go to the other extreme and use such dilute substrate solutions that the rate of enzyme action follows the die-away curve, and falls parallel to the substrate concentration. The die-away "monomolecular" formula can be applied. Its constant will vary directly as the amount of active enzyme present. This use of dilute substrate concentrations has an advantage when the substrate is a rare or expensive substance. Furthermore, this procedure may be forced on one if the substrate is not soluble enough to permit a concentrated solution to be made such as is necessary to drive the reaction to its full speed. Bergmann, Fruton and their collaborators (2) in studies of pepsidases have employed dilute peptide concentrations and measured the activities by the monomolecular constant.

The degree of dilution of substrate that is necessary to slow the enzyme down and make the activity a linear function of the substrate concentration will be varied by whatever factors affect the "combining constant" that we have discussed. The slower the combining phase of the enzyme action the higher will be the range of substrate concentrations over which

this phase consumes nearly all the time, and over which in consequence the rate of action is directly proportional to substrate concentration. We have seen in the case of urease that lowering the pH by 1 unit diminishes the combining velocity constant to $1/11$ th. Also, increasing the salt content of the solution retards the combining phase. Invertase behaves qualitatively like urease, with regard to pH . In a given concentration range of sucrose invertase may, depending on the pH , either follow the die-away formula, or on the contrary (at higher pH) it may be independent of this formula and show a speed not increased by rise in sucrose concentration.

3. *Activity Measurement by Use of the Inverse Time-Enzyme Relation*

Finally, for measuring enzyme activity one may use the general law that, with a given substrate solution, the time required to decompose a given fraction, say half, of the substrate will vary inversely as the amount of enzyme present. This law holds whether the reaction starts with such a high substrate concentration that the enzyme works at full speed, or such a low concentration that the die-away formula holds, or at an intermediate concentration between the two. The law also holds regardless of how the products affect the activity of the enzyme, because the same amount of products will form in all cases if the same amount of substrate is hydrolyzed. One condition under which this time method fails, however, is that which occurs when the enzyme loses activity during the reaction. This factor is likely to be important. It would cause an enzyme preparation of low activity, requiring a long time to split half the substrate, to give results indicating even less activity than is present, because during the long digestion period much of the enzyme might disappear. In an extreme case the enzyme might all disappear before half the substrate was decomposed. The use of the time method therefore depends more than either of the other procedures on *stability* of the enzyme during its action on the substrate. The method works very well with crude, stable urease, which can be estimated by it even without adding buffers to prevent the alkalization that accompanies the change of urea into ammonium carbonate. On the other hand, the method cannot well be used with arginase because, even when protected by manganese, arginase loses activity under the conditions of its action, and the longer times required by a relatively dilute arginase preparation would involve more enzyme destruction than the shorter times required by a stronger preparation.

It is obvious that no set rule can be formulated for the technique of measuring enzyme activity. One must know the behavior of the enzyme

one is seeking to study and fit the technique to its stability, the influence of pH, temperature, and other factors. Nevertheless it appears that the two-phase law of enzyme action may assist in understanding and choosing the conditions that are best adopted to activity measurement in a given case.

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A CLASSIFICATION OF PROTEOLYTIC ENZYMES¹

By

MAX BERGMANN

New York, N. Y.

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I. Introduction

It is a matter of general agreement that proteolytic enzymes hydrolyze peptide bonds and that this hydrolysis is a reversible process. The fact is also recognized by all authorities that every proteolytic enzyme discriminates between various types of peptide bonds and that this specificity comes into play in the course of the physiological digestion of proteins. Every attempt to classify the proteolytic enzymes on the basis of their specificity must suffer from the fact that our knowledge of the characteristics of the specificity of proteolytic enzymes is of a rather recent date and is, at the present time, still in a state of active development.

Emil Fischer was the first to investigate the specificity of proteolytic enzymes by their action upon several peptides (1). Table I is taken from a paper published by Fischer and Abderhalden in 1905 describing the action of pancreatic juice upon 29 synthetic substrates. These workers concluded from their experiments that the enzymatic sensitivity of a peptide is a function of the number, constitution, configuration and sequence of the amino acid residues that are present in the peptide. Fischer expressed the hope that a closer study of the action of enzymes upon peptides might provide a means to classify the synthetic peptides according to their biological significance. Fischer did not consider, however, the use of the various peptides for the classification of the pro-

¹ Some of the material in this paper was presented on June 26, 1941, at the Protein Research Conference held at Stanford University, California.

teolytic enzymes and, as a matter of fact, left open the question of whether the pancreas gland secretes only one or several proteolytic enzymes. It may be anticipated here, that our present knowledge of the specificities of trypsin and chymotrypsin permits us to conclude that neither of these enzymes can be responsible for the enzymatic hydrolysis of peptides observed in Fischer's experiments. On the other hand, the individual enzymes responsible for most of these splittings are not yet recognized.

TABLE I

ACTION OF PANCREATIC ENZYMES UPON SEVERAL PEPTIDES
(E. Fischer and E. Abderhalden, *Z. physiol. Chem.*, **46**, 52 [1905])

Hydrolysed	Not hydrolysed
Alanylglycine	Glycylalanine
Alanylalanine	Glycylglycine
Alanyl-leucine A	Alanylleucine B
Leucylisoserine A	Leucylalanine
Glycyl- <i>L</i> -tyrosine	Leucylglycine
Leucyl- <i>L</i> -tyrosine	Leucylleucine
Alanylglycylglycine	Aminobutyrylglycine
Leucylglycylglycine	Aminobutyrylaminobutyric acid A
Glycylleucylalanine	Aminobutyrylaminobutyric acid B
Alanylleucylglycine	Aminoisovalerylglycine
Dialanyleystine	Glycylphenylalanine
Dileucyleystine	Leucylproline
Tetraglycylglycine	Diglycylglycine
Triglycylglycine ester	Triglycylglycine
	Dileucylglycylglycine

Fischer's results were later rejected by Waldschmidt-Leitz (2) and attributed to the poor and variable quality of the enzyme preparations he employed. Waldschmidt-Leitz concluded from his specificity experiments with purified preparations of proteolytic enzymes that a qualitative differentiation of the peptides of the natural-occurring amino acids on the basis of their sensitivity toward pancreatic enzymes is not possible. In his opinion, proteolytic enzymes do not distinguish between the peptides of different amino acids but rather those of different chain length. This latter specificity theory was further developed in many details by the Willstätter school of thought, especially by Grassmann (3) and Waldschmidt-Leitz and resulted in the classification of the proteolytic enzymes, presented in Table II. This classification distinguishes between proteinases that are supposed to attack high molecular proteins only, and peptidases that attack the low molecular peptides. The group of peptidases was believed to contain a dipeptidase, an aminopeptidase and two carboxypeptidases, in addition to a few special enzymes such as prolinase and prolidase. The peptidases were assumed to combine with a terminal α -amino group or a terminal α -carboxyl group of the substrate (4). It was thought that this combination of the enzyme with one of the aforementioned atomic groups of the substrate rendered the adjacent peptide linkage of the substrate more labile toward hydrolysis. The specificity of the various proteinases was supposed to be adapted to the anionic, cationic or zwitterionic character of the substrates. The proponents of this

specificity concept did not consider the possibility that proteolytic enzymes might participate actively in the physiological synthesis of proteins. It appears incomprehensible, indeed, that enzymes specifically adapted to the chain length of their substrates should be capable of organizing the intricate pattern of a genuine protein.

TABLE II
CLASSIFICATION OF PROTEOLYTIC ENZYMES
(as generally accepted about 1935)

Enzyme	Substrate	Specificity
A. Peptidases		
Dipeptidase	Dipeptides	$NH_2\cdot CHR\cdot CO- -NH\cdot CHR\cdot COOH$
Aminopolypeptidase	Polypeptides	$NH_2\cdot CHR\cdot CO- -NH\cdot \dots\dots$
Tryptic carboxypolypeptidase		} $\dots\dots CO- -NH\cdot CHR\cdot COOH$
Catheptic carboxypolypeptidase		
B. Proteinases		
Pepsin	High molecular proteins and peptones	Cations
Trypsin		Anions
Papain and cathepsin		Zwitterions

Other enzymes: Prolinase, prolidase, dehydro-dipeptidase, gelatinase, yeast-trypsin.

It has always appeared to the author that the specificity concept summarized in Table II rested upon too narrow an experimental base with respect to the enzymes investigated as well as with regard to the substrates employed. The enzymes, frequently obtained by glycerin extraction of animal tissues and purified by the adsorption-elution method, in many cases represented only a fraction of the enzymatic activity of the original tissues² and were, in general, not enzymatically homogeneous. The substrates studied contained almost exclusively the three monoamino acids glycine, alanine and leucine, while the majority of the 25 amino acids which are known as constituents of proteins are of greater structural complexity.

The situation with respect to the substrates was improved in 1932 when a method was devised that permitted the synthesis of optically active peptides of the more complicated amino acids (6). Enzymatic studies performed with the help of such peptides necessitated a revision of the specificity concept and of the classification of proteolytic enzymes. This revision is at present still under way and will be, in all probability, for some

² Grassmann, Volmer and Windbichler have demonstrated that "dipeptidase" loses the major part of its activity toward leucylglycine when subjected to the usual adsorption routine by adsorption on aluminum hydroxide C_γ and subsequent elution (5).

time to come. Nevertheless, it is already feasible to give a preliminary outline of the specificity requirements of several enzymes and to tentatively classify the proteolytic enzymes on the basis of their specificity requirements.

II. Tentative Classification of Proteolytic Enzymes—Homospecificity and Heterospecificity

First, it may be mentioned that the existence of an individual enzyme that is adapted to the hydrolysis of the dipeptides of the natural amino acids and of dipeptides only, has become somewhat uncertain. The ability to hydrolyze dipeptides has frequently been found to be associated with an activity toward polypeptides. Thus, crystallized carboxypeptidase splits the dipeptide *l*-tyrosyl-*l*-tyrosine (7). Similarly, an aminopeptidase contained in beef spleen extracts was shown to hydrolyze *l*-leucylglycine as well as *l*-leucineamide (8).

Using the intestinal mucosa of higher animals, the classical source for "dipeptidase," a rather indicative experiment may be performed (9). Crude aqueous extracts of swine intestinal mucosa are highly active toward glycylglycine, alanylglycine, leucylglycine, leucylglycylglycine, leucineamide and many other peptides. If such mucosa extract is precipitated with acetone and the precipitate fractionated with ammonium sulfate, an enzyme preparation may be obtained which no longer has any activity toward the dipeptides glycylglycine and alanylglycine, but still possesses high activity toward the dipeptide leucylglycine, the tripeptide leucylglycylglycine and leucineamide.¹ This demonstrates that the enzyme that hydrolyzes the dipeptide leucylglycine does not attack the other previously mentioned dipeptides but is active toward other leucine derivatives.

Apparently, the specificity of the hitherto known peptidases is not adapted to the structural differences between dipeptides and polypeptides, but is adapted to the nature of the so-called side groups, *i. e.*, the atomic groups that represent the differences between the various α -amino acids. Consequently, one must expect to find in the future not only one but several aminopeptidases which should differ in their side group specificity and, similarly, to encounter several carboxypeptidases of distinct side group specificity.

Many of the enzymes that previously had been regarded as dipeptidases simply because they were found to hydrolyze leucylglycine or similar dipeptides will in the future be recognized as aminopeptidases or carboxy-

¹ This enzyme might belong to the group of leucylpeptidases studied by Linderstrøm-Lang (25) and by Berger and Johnson (18).

peptidases. It cannot be predicted how many enzymes, if any, will remain to be classified as dipeptidases. The information that is available at the present time neither proves nor disproves the existence of an enzyme or a class of enzymes that is specifically adapted to the exclusive hydrolysis of dipeptides.

The side group specificity of the proteinases was first demonstrated for the gastro-intestinal enzymes trypsin (10), chymotrypsin (11) and pepsin (12) and more recently also for the intracellular enzymes or cathepsins (8). For each of these proteinases there could be found several low molecular substrates containing the requisite side groups. Many of these low molecular substrates are hydrolyzed by proteinases with a speed of the same order of magnitude as that observed in the enzymatic hydrolysis of genuine proteins (13). Thus, it must be concluded that the specificity of the proteinases cannot be restricted or adapted to high molecular substrates.

On inspection of the revised classification of proteolytic enzymes, as presented in Table III, it will be noticed that it is based on two characteristics of the proteolytic specificity, namely, that each proteolytic enzyme requires the presence of certain atomic groupings within the so-called backbone and within the side chain of the substrate molecule. In order to determine for each enzyme the nature of these requisite groups, it was hitherto necessary to study the action of the enzyme upon a considerable number of synthetic substrates, the structures of which were systematically modified. The indispensable groups within the backbone of the substrate are italicized in Column 2 of Table III. The requisite side groups of the substrates for each enzymatic specificity are present in a precisely defined structural relation to the sensitive peptide bond. The location of the side groups is marked by the letter *R* in the formulas of Column 2, and their detailed structural formulas are given in Column 3.

It is indicated in Table III that the substrates for the aminopeptidases contain as requisite groups in the backbone of the substrate an amino group in addition to the carbonyl of the sensitive peptide bond and in close proximity to it. The carboxypeptidases, on the other hand, require in their substrates a carboxyl group in addition to the imido group of the attacked or synthesized peptide bond. The hydrolytic action of both aminopeptidases and carboxypeptidases upon their substrates invariably leads to the production of free amino acids.

When proteinases hydrolyze or synthesize a peptide bond, they require, in proximity to their point of action, another peptide bond. Thus, substrates hydrolyzed by pepsin, trypsin or chymotrypsin contain a second

TABLE III
TENTATIVE CLASSIFICATION OF PROTEOLYTIC ENZYMES BY THEIR SPECIFICITY

Enzyme	Requisite groups in backbone of substrate and mechanism of catalysed reaction	Requisite groups in side chain of substrate $R =$
(1)	Peptidases (Exopeptidases) (2)	(3)
Leucine-aminopeptidase from intestinal mucosa Aminopeptidases from spleen and kidney (Cathepsins III) Other aminopeptidases	$\left. \begin{array}{l} R \\ \\ NH_2-CH-CO-NH \dots \\ \\ R \end{array} \right\} \rightleftharpoons \left. \begin{array}{l} R \\ \\ NH_2-CH-COOH + NH_2 \\ \text{Ditto} \end{array} \right\}$	$\begin{array}{c} CH_2 \\ \diagup \quad \diagdown \\ CH-CH_2 \dots \\ \diagdown \quad \diagup \\ CH_2 \end{array}$
Carboxypeptidase from pancreas Carboxypeptidases from spleen and kidney (Cathepsins IV) Other carboxypeptidases	$\left. \begin{array}{l} R \\ \\ \dots CO-NH-CH-COOH \\ \\ R \end{array} \right\} \rightleftharpoons \left. \begin{array}{l} R \\ \\ \dots COOH + NH_2-CH-COOH \\ \text{Ditto} \end{array} \right\}$	$\begin{array}{c} HO-CH_2 \dots \\ \\ \text{Cyclohexane ring} \end{array} \quad \text{or} \quad \begin{array}{c} CH_2 \dots \\ \\ \text{Cyclohexane ring} \end{array}$

Proteinases (Endopeptidases)

Pepsin	$\begin{array}{c} \text{.....CO-NH-CHR-CO-NH-CH.....} \\ \updownarrow \\ \text{.....CO-NH-CHR-COOH + NH}_2\text{-CH.....} \end{array}$	$\begin{array}{c} \text{HO} \text{---} \text{Cyclohexyl} \text{---} \text{CH}_2 \text{.....} \\ \text{or} \\ \text{Cyclohexyl} \text{---} \text{CH}_2 \text{.....} \end{array}$
Trypsin Trypsinases from spleen and kidney (Cathepsins II)	$\begin{array}{c} \text{R} \\ \\ \text{CO-NH-CH-CO-NH} \\ \updownarrow \\ \text{CO-NH-CH-COOH + NH} \end{array}$	$\begin{array}{c} \text{NH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{.....} \\ \text{or} \\ \text{NH}_2\text{-C(NH}_2\text{)-CH-CH}_2\text{-CH}_2\text{.....} \end{array}$
Chymotrypsin*	$\begin{array}{c} \text{R} \\ \\ \text{.....CO-NH-CH-CO-NH.....} \\ \updownarrow \\ \text{.....CO-NH-CH-COOH + NH.....} \end{array}$	$\begin{array}{c} \text{HO} \text{---} \text{Cyclohexyl} \text{---} \text{CH}_2 \text{.....} \\ \text{or} \\ \text{Cyclohexyl} \text{---} \text{CH}_2 \text{.....} \end{array}$

* Recent observations seem to indicate the presence in chymotrypsin of several enzymatic specificities (J. S. Fruton and M. Bergmann, unpublished data). The above description, therefore, covers only one of these specificities.

peptide bond in direct proximity to the carbonyl group of the hydrolyzed peptide bond.

From what has been said before, it is obvious that both classes of enzymes, peptidases and proteinases are concerned with the hydrolysis and synthesis of peptide bonds and that both classes of enzymes are capable of acting upon low molecular peptides. However, the class comprising the aminopeptidases and carboxypeptidases acts upon terminal peptide bonds only and, therefore, can be termed *exopeptidases* (14). The other class comprising enzymes such as pepsin, trypsin, chymotrypsin and others is capable of attacking centrally located peptide bonds as well as terminal peptide bonds and has therefore been designated as *endopeptidases*. The terms *exopeptidases* and *endopeptidases* are in good agreement with the specific requirements of the two classes of enzymes, while the terms *peptidases* and *proteinases* have their origin in a specificity concept that is now recognized as erroneous.

With regard to the side group specificity, it may be stated that pepsin and chymotrypsin require the presence in their substrates of a tyrosine or phenylalanine residue, *i. e.*, a *p*-hydroxylbenzyl or benzyl side chain, while trypsin requires the side groups characteristic for arginine or lysine residues. In the case of trypsin and chymotrypsin, the characteristic side group is located between the requisite backbone groups; in the case of pepsin, it is located outside the requisite groups of the backbone.

Of special interest with respect to the problem of protein synthesis are the intracellular proteolytic enzymes. Therefore, in the author's laboratory there was recently begun a study of the specificities of the proteolytic enzymes of animal tissues such as spleen and kidney. These organs were found to contain a surprisingly large number of proteolytic enzymes. An extract of beef spleen, for instance, was found to contain at least three proteinases, an aminopeptidase and a carboxypeptidase. As yet, none of these enzymes has been isolated in pure form. However, the various individual enzymes contained in a mixture such as a tissue extract can be distinguished from one another and the specificity of each enzyme can be characterized by a method developed recently (15). This method involves the study of the action of the enzyme mixture upon a number of synthetic substrates, each of which is hydrolyzed by the enzyme preparation with the scission of only one peptide linkage per substrate molecule. In proteolytic experiments performed with such substrates, first order reaction kinetics are frequently obtained and the numerical values of the reaction rates are found to be a direct measure of the enzyme concentration. It is frequently observed that the reaction rates for the hydrolyses of two substrates are

affected to a different degree when the enzyme preparation under study is subjected to purification procedures. If such is the case, it may be concluded that the observed hydrolyses of the two substrates are effected by two or more enzymes. By such a systematic quantitative study of the action of beef spleen extracts upon a number of synthetic substrates, it has become possible to differentiate the previously mentioned five spleen enzymes.

One of these spleen enzymes—designated as beef spleen cathepsin I or beef spleen pepsinase—hydrolyzes the typical pepsin substrates. Moreover, the spleen pepsinase requires the same groups in the backbone of the substrate and has the same side group specificity as pepsin. However, despite these similarities, the spleen pepsinase is by no means identical with pepsin. That pepsin and spleen pepsinase are two different individual enzymes is convincingly demonstrated by the fact that even rather impure preparations of spleen pepsinase have a much greater proteolytic activity per milligram of protein nitrogen than that of crystalline pepsin. The pH optima of the two enzymes toward identical substrates are different—5.4 and 4.0, respectively, for the hydrolysis of carbobenzoxy glutamyl tyrosine by the spleen pepsinase and by pepsin. Beef kidney also yields a cathepsin I, *i. e.*, a pepsinase possessing a specificity similar to that of pepsin and of beef spleen pepsinase. We are therefore confronted with the remarkable fact that various animal organs adapted to such different physiological functions as are the fourth stomach, spleen and kidney of cattle, produce enzymes which exhibit an identical specificity type (16).

Other examples of enzymes possessing similar specificities are beef spleen cathepsin II, beef kidney cathepsin II and swine kidney cathepsin II. All three enzymes exhibit the same type of specificity and therefore split the same synthetic substrates as does trypsin. These enzymes may therefore be designated as beef spleen trypsinase, beef kidney trypsinase and swine kidney trypsinase. We have, at present, no method at our disposal to ascertain whether or not the three catheptic trypsinases are identical enzymes. There can be no doubt, however, that the catheptic trypsinases, on the one hand, and trypsin, on the other, are different chemical entities. The spleen and kidney trypsinases, in contrast to trypsin, act upon their substrates only in the presence of an activator. Moreover, the pH optimum of the catheptic trypsinases is 4.9; that of trypsin is 7.8.

The observation that several enzymes may resemble one another with regard to side group and backbone specificity is not restricted to proteinases. Analogous similarities were observed for the specificities of the pancreatic carboxypeptidase and the catheptic carboxypeptidases (*i. e.*,

cathepsins IV) of beef spleen, beef kidney and swine kidney; moreover, for the specificities of the catheptic leucine-aminopeptidases (*i. e.*, cathepsins III) of spleen and kidney and of the leucine-aminopeptidase from intestinal mucosa. Thus, enzymes of undoubtedly different chemical individuality may correspond to a similar type of backbone and side group specificity and may, therefore, occupy identical places in the scheme of classification presented in Table III. One might speak of the members of such a group of enzymes as "homospecific" enzymes in contrast to "heterospecific" enzymes, *i. e.*, enzymes exhibiting differences of the backbone or side group specificity. For example, trypsin and beef spleen trypsinase are homospecific, while trypsin is heterospecific when compared with chymotrypsin.

The phenomenon of homospecificity has been placed on a quantitative basis by comparing the reaction kinetics of the action of homospecific and of heterospecific enzymes upon several synthetic substrates (16). In Table IV each of the five homospecific enzymes—trypsin, beef spleen trypsinase,

TABLE IV
HYDROLYSIS OF BENZOYL-*L*-ARGININEAMIDE AND BENZOYL-*L*-LYSINEAMIDE BY SEVERAL HOMOSPECIFIC ENZYMES

Enzyme	Temperature, ° C.	Activator	pH	$C \times 10^3$		$\frac{C_{BAA}}{C_{BLA}}$
				Benzoyl-arginine-amide	Benzoyl-lysine-amide	
Beef spleen trypsinase	40	Cysteine	4.7	8.3	3.8	2.2
Beef kidney trypsinase	40	Cysteine	4.7	8.7	3.7	2.3
Swine kidney trypsinase	40	Cysteine	4.7	27	11	2.5
Trypsin	25	None	7.4	42	20	2.1
Papain trypsinase	40	Cysteine	5.0	167	78	2.1

The symbols used in describing the proteolytic coefficient C and the proteolytic quotient for the substrates benzoylarginineamide and benzoyllysineamide— C_{BAA}/C_{BLA} —have been defined in (15).

beef kidney trypsinase, swine kidney trypsinase and the trypsinase contained in papain—were tested with the two substrates benzoyl-*L*-arginineamide and benzoyl-*L*-lysineamide. The experimental conditions were such that good first order reaction constants were obtained with both substrates and with each of the enzymes. These reaction constants for enzyme concentrations of 1 mg. of protein N per ccm. of test solution are the so-called proteolytic coefficients C , reported in Table IV for the action of the five enzymes upon benzoyl-*L*-arginineamide (C_{BAA}) and upon benzoyl-*L*-lysineamide (C_{BLA}). The quotient C_{BAA}/C_{BLA} , as given in the last vertical

column of Table IV, compares the reaction rates of the enzymatic hydrolysis of the two substrates or, in other words, states the change of the numerical value of the reaction rate produced when the arginine residue of the first substrate is replaced by the lysine residue in the second substrate. It will be noted that this quotient was found to be 2.1 for trypsin, also 2.1 for papain trypsinase, and that almost identical values were obtained for the spleen and kidney trypsinases.

In Table V the homosppecific carboxypeptidases from beef pancreas, beef spleen, beef kidney and swine kidney are compared with respect to their action upon the substrates carbobenzoxyglycyl-*l*-phenylalanine and carbobenzoxyglycyl-*l*-tyrosine. In all instances the proteolytic quotient C_{CGP}/C_{CGT} is found to be 1.6 to 1.8.

TABLE V
COMPARISON OF SEVERAL HOMOSPECIFIC CARBOXYPEPTIDASES

Enzyme	Temperature, ° C.	Activator	pH	$C \times 10^3$		$\frac{C_{CGP}}{C_{CGT}}$
				Carbobenzoxy-glycyl- <i>l</i> -phenylalanine	Carbobenzoxy-glycyl- <i>l</i> -tyrosine	
Beef spleen carboxypeptidase	40	Cysteine	5.0	2.5	1.5	1.7
Beef kidney carboxypeptidase	40	Cysteine	5.1	6.3	4.0	1.6
Swine kidney carboxypeptidase	40	Cysteine	5.0	34	19	1.8
Beef pancreas carboxypeptidase	25	None	7.7	6570	3620	1.8

In Table VI there is reported a comparison of the action of the three pepsinases from spleen and kidney as tested with the aid of the two substrates carbobenzoxy-*l*-glutamyl-*l*-phenylalanine and carbobenzoxy-*l*-glutamyl-*l*-tyrosine. The proteolytic quotients C_{CGP}/C_{CGT} were found to be 0.48, 0.42 and 0.50.

TABLE VI
COMPARISON OF SEVERAL PEPSINASES

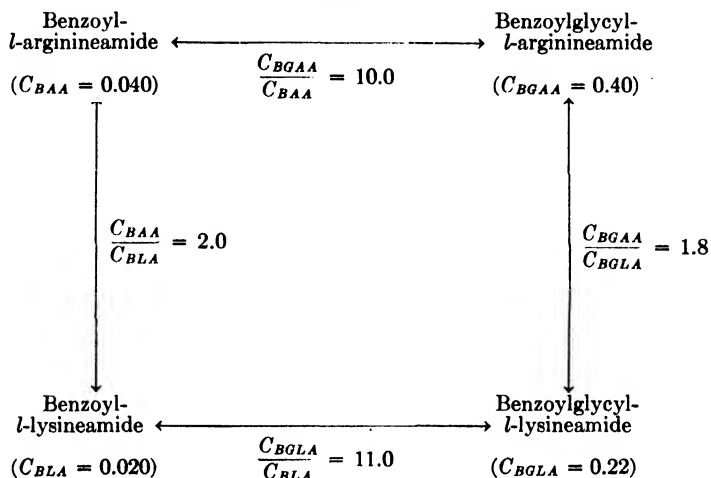
Enzyme	Temperature, ° C.	Activator	pH	$C \times 10^3$		$\frac{C_{CGP}}{C_{CGT}}$
				Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -phenylalanine	Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -tyrosine	
Beef spleen pepsinase	25	None	5.4	1.6	3.3	0.48
Beef kidney pepsinase	25	None	5.4	0.84	2.0	0.42
Swine kidney pepsinase	25	None	5.4	1.6	3.2	0.50

The carboxypeptidases of Table V and the pepsinases of Table VI have the same side group specificity in that both require the side groups of a phenylalanine or a tyrosine residue. On the other hand, the two substrates discussed in Table V differ from one another only with respect to these side groups—the phenylalanine residue of one substrate is replaced by a tyrosine residue in the other substrate and the same difference exists between the two substrates discussed in Table VI. However, while this modification of the substrates effects a retardation of the carboxypeptidase action, it accelerates the action of the pepsinases. One arrives, then, at the following conclusion: Homospecific enzymes respond similarly, heterospecific enzymes respond differently when the characteristic side group of the substrate is altered. Thus, the proposed classification of the proteolytic enzymes by their specificity requirements finds support in a quantitative comparison of the reaction kinetics of the enzymes belonging to the various subgroups. Conversely, it will become possible in the future to assign to a proteolytic enzyme of unknown specificity its proper place within the system by studying the kinetics of its action upon a few well-chosen synthetic substrates and by thus determining whether this enzyme is homospecific or heterospecific with respect to other enzymes of known specificity.

In the previously discussed examples, the homospecificity of a group of enzymes was concluded from proteolytic quotients which were in every case determined with the aid of two substrates that differed only with respect to the nature of their characteristic side groups. Thus, the two substrates benzoylarginineamide and benzoyllysineamide had been employed for the determination of proteolytic quotients of the trypsinases. The question might be asked whether a fundamentally different value of the proteolytic quotient would have resulted if the two substrates benzoylglycyl-*L*-arginineamide and benzoylglycyl-*L*-lysineamide, which also contain arginine and lysine residues as characteristic side groups, had been employed. It will be noted from Fig. 1 that the rate of trypsin action upon benzoylglycylarginineamide is ten times greater than that of trypsin action upon benzoylarginineamide, C_{BGAA}/C_{BAA} being equal to 10.0. However, a similar ratio results for the action of trypsin upon benzoylglycyllysineamide and benzoyllysineamide, C_{BGLA}/C_{BLA} being 11.0. As a consequence, the proteolytic quotient C_{BGAA}/C_{BGLA} is almost identical with the quotient C_{BAA}/C_{BLA} .

The above data demonstrate that both the action of a proteolytic enzyme upon various substrates and the action of various homospecific enzymes upon identical substrates are sometimes interrelated by quantitative rules of a surprisingly simple nature. In such cases it becomes pos-

Fig. 1.—Proteolytic Coefficients and Quotients for the Action of Trypsin upon Several Substrates



sible to calculate the approximate rates of certain enzymatic reactions before they have been studied experimentally. If, for instance, the proteolytic coefficient for the hydrolysis of benzoylarginineamide by a preparation of beef spleen trypsinase is known, then one may calculate the approximate rates at which this enzyme preparation will hydrolyze the substrates benzoylglycylarginineamide and benzoylglycyllysineamide. If, on the other hand, the unknown substrate benzoyl-*l*-alanyl-*l*-arginineamide were synthesized and its hydrolysis by pancreatic trypsin were studied, then one could predict, on this basis, the reaction rate with which trypsin would hydrolyze the also unknown substrate benzoyl-*l*-alanyl-*l*-lysineamide.

III. The Activation of Proteolytic Enzymes

The classification of the proteinases and peptidases, as suggested in Table III, is based on the specificity of the enzymes as expressed in structural requisites to be found in the substrates. It should be our goal to proceed from these symptoms of the enzymatic specificity to its origin, namely, the constitution of the various proteolytic enzymes. For example, one would like to learn which constitutional properties of the various proteolytic enzymes are responsible for their specificities and, in particular, for their differences in specificity, and whether there are certain constitu-

tional properties common to a group of homospecific enzymes that might account for the similarity of their specificity requirements. At present such questions cannot be answered since our knowledge of the constitutional details of proteolytic enzymes is almost nil. There is only one constitutional property that we know to be common to many proteolytic enzymes and that has frequently been discussed in connection with their classification. It consists in the aforementioned fact that many proteolytic enzymes are active only after they have been activated by HCN or sulfhydryl compounds; others are active only when activated by metals. The activation by HCN and sulfhydryl compounds is frequently—and, in the author's opinion, erroneously—regarded as a reduction of the general type described schematically in Table VII.

TABLE VII
THE ACTIVATION OF INTRACELLULAR ENZYMES
Oxidation-Reduction Theory

2(Enz-SH)	$\begin{array}{c} \xrightarrow{\text{Oxidation}} \\ \xleftarrow{\text{Reduction}} \end{array}$	Enz S—S Enz
(Proteolytically active)		(Proteolytically inactive)

On the other hand, a group of enzyme chemists (17) have advanced the hypothesis that all proteolytic enzymes have a dualistic nature in that they consist of a colloidal protein, acting as carrier, and a specific active part of unknown chemical nature. On the basis of recent experiments, it appears that both theories—the activation theory and the dualistic theory—when properly modified, might be combined to include all the activatable proteolytic enzymes that are known at the present time.

It is known from the experiments of Johnson (18) and of Maschmann (19) that the activity of the intestinal enzyme that hydrolyzes *l*-leucylglycine increases considerably on the addition of manganese or magnesium salts. This enzyme is also known to be inhibited by HCN or H₂S (20). These properties may be taken as indications that the active enzyme is a dissociable metal-protein compound. Indeed, it has recently been observed (21) that on dialysis of the active enzyme a preparation is obtained which is completely inactive toward *l*-leucylglycine but regains a high activity on addition of manganese or magnesium salts. This reactivation is a time reaction and the degree of the final activity depends upon the concentration of the metal ions added. Several other proteolytic enzymes of

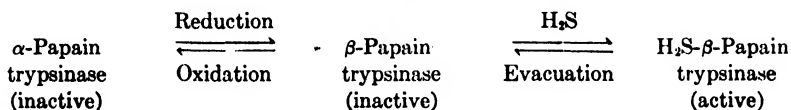
the intestines and many bacterial enzymes investigated by Maschmann (19) behave similarly.

The activation of the intracellular enzymes of plants and animals by HCN and sulfhydryl compounds is complicated by the fact that these enzymes are almost always accompanied by sulfur-containing compounds which are sometimes referred to as natural activators. Recently papain and cathepsin preparations have been obtained that contain no natural activators. Such a purified papain preparation was found to be completely inactive toward the substrate benzoylarginineamide and to remain inactive after HCN had been added (Table VIII). This is somewhat surprising since ordinary inactive papain preparations contain an enzyme—it was called papain trypsinase in Table IV—which can be activated by HCN and then will hydrolyze benzoylarginineamide, benzoyllysineamide and similar substrates. The indifference of the purified papain trypsinase toward HCN is the more surprising since the HCN activation of papain is generally regarded as typical for this whole group of activation phenomena. The purified papain trypsinase which is inactive and not activated by HCN and which, for the sake of convenience, may be called papain α -trypsinase, may be activated by H_2S . When the H_2S is subsequently removed *in vacuo*, a second inactive form—papain β -trypsinase—is obtained. In contrast to α -trypsinase, β -trypsinase can be activated by HCN. Thus, papain trypsinase exists in two inactive forms: one that cannot be activated by HCN, and one that can.

TABLE VIII

REVERSIBLE ACTIVATION OF PAPAIN TRYPSINASE
Transformation of α - to β -Trypsinase by Traces of SH-Compounds
Substrate: Benzoyl-L-arginineamide

Activator, mM per cc.	Hydrolysis of substrate K (first order)
(a) None	0.0000
(b) HCN (0.02)	0.0000
(c) H_2S (0.003)	0.0019
(d) (c) evacuated	0.0003
(e) Cysteine (0.00004)	0.0000
(f) (e) + HCN (0.02)	0.0020
(g) (f) evacuated	0.0000



We assume that the above-mentioned action of H_2S upon papain trypsinase consists of two steps, namely, first the transformation of α -trypsinase into β -trypsinase and, second, the activation of β -trypsinase. Both steps are effected by H_2S . The second step, the activation of β -trypsinase by H_2S , may be reversed by evacuation. By the same procedure, the activation of β -trypsinase by HCN may be reversed. The reversibility of these activations shows that they consist in the formation of the two dissociable compounds, $\text{HCN-}\beta$ -trypsinase and $\text{H}_2\text{S-}\beta$ -trypsinase. HCN and H_2S play for papain β -trypsinase the same role as do manganese and magnesium in the activation of many intestinal and bacterial enzymes. With respect to the α - and β - forms of papain trypsinase, it seems probable that β -trypsinase is the reduced form of α -trypsinase and may be reversed to α -trypsinase by oxidation. However, it should be understood that the reduction of α -papain to β -papain is not an activation, since both forms are enzymatically inactive toward benzoylarginineamide.

The activable spleen trypsinase was also found to exist in two inactive forms and to be activated through the formation of dissociable activator- β -trypsinase compounds. This appears to indicate that the H_2S -activable intracellular enzymes of the higher animals follow an activation pattern similar to that discussed for the plant enzyme, papain trypsinase.

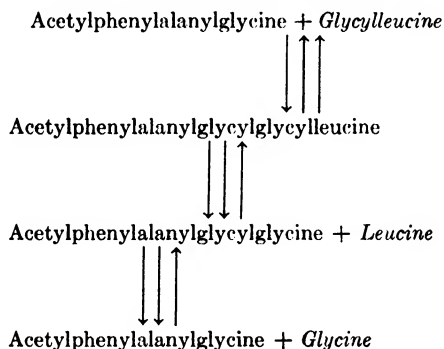
Hence, two mechanisms of activation of proteolytic enzymes are known: the activation by metals and the activation by sulfhydryl compounds or HCN . Both these activation processes have the common characteristic that the active enzyme is composed of an inactive apoenzyme and an activator.

Thus far, the structure of the activated enzymes agrees with the theory of the dualistic nature of proteolytic enzymes. However, specificity studies do not support the claim that the protein part of the dualistic enzyme is merely a colloidal carrier for another and supposedly active and specific part of the enzyme. On the contrary, the protein part of the activated enzymes exclusively determines the character of the specificity of the activated enzyme.

IV. Coupled Reactions as Induced by Proteolytic Enzymes

The classification of proteolytic enzymes by their specificities has its basis in experiments in which the enzymes act upon simple substrates of known structure. The fact that a substrate is attacked by an enzyme has always been taken as an unequivocal indication that the structure of the substrate falls within the specificity range of the enzyme. However, recent

experiments have demonstrated that such reasoning does not always conform to the truth. Peptides which are perfectly stable in contact with a certain proteolytic enzyme are sometimes attacked by the same enzyme after a second peptide has been added to the mixture. To cite the best known example of this type of enzymatic action, glycyl-*L*-leucine is not hydrolyzed by cystine-activated papain, but is hydrolyzed when acetylphenylalanylglycine is simultaneously present in the test solution (22). It could be shown that under these conditions the enzymatic hydrolysis of glycylleucine takes an indirect course and represents the result of three distinct enzymatic reaction steps. The first step consists in a combination of glycyl-*L*-leucine and acetylphenylalanylglycine resulting in the formation of the acetylated tetrapeptide, acetylphenylalanyldiglycyl-*L*-leucine. From this tetrapeptide, first leucine and subsequently one glycine residue are split off, thus regenerating acetylphenylalanylglycine.



In this indirect hydrolysis of glycylleucine an enzyme, or rather a mixture of enzymes (papain, as obtained from the latex of *carica papaya* contains a considerable number of proteolytic enzymes⁴) attacks a substrate which is clearly outside the specificity limits of all the enzymes present. This transgression of the specificity limits is made possible through the presence of a so-called cosubstrate, that is, a second peptide (acetylphenylalanylglycine) that does not appear in the over-all equation of the reaction. The over-all reaction, the hydrolysis of glycylleucine, is the sum of three reaction steps which are coupled to one another.

Coupled reactions of the type just described are not rare exceptions but take place rather frequently when two or more peptides are in contact with proteolytic enzymes. It has repeatedly been observed (22) that peptides

⁴ Two crystalline enzyme preparations obtained from the latex of *carica papaya* have been designated papain and chymopapain (23).

that are resistant to certain proteolytic enzymes are hydrolyzed by the same enzymes after hemoglobin, or casein, or blood serum has been added to the peptide-enzyme mixture.

When proteolytic enzymes have at their disposal a number of peptides, as will be the case in the course of an enzymatic hydrolysis of a protein, the possibility will frequently arise for the occurrence of a sequence of coupled reactions—synthetic as well as hydrolytic reactions—the over-all result of which might give a misleading picture of the specificity of the enzymes involved. All attempts in the past to study the specificity of proteolytic enzymes by using proteins or mixtures of protein split products as substrates have unwittingly neglected this possibility.

The course of a sequence of coupled proteolytic reactions must depend upon the specificities of the enzymes involved and upon the constitution of the peptides present. Consequently, if a given proteolytic system consisting of several enzymes and peptides is augmented by the addition of a foreign peptide or protein, the fate of the proteolytic system will, in all probability, be changed by the establishment of a sequence of coupled reactions in which the foreign peptide participates.

The sequence of coupled proteolytic reactions in the various tissues of an organism can follow its normal course only when it is protected against interferences by intruding foreign peptides or proteins. The presence within the gastro-intestinal tract of a multitude of proteolytic enzymes each of which exhibits a highly developed specificity may be explained as a mechanism providing such a protection against foreign peptides containing undesirable combinations of amino acids.

It is well known that the intrusion or introduction of a foreign protein into an organism by ways other than the oral route results in a number of characteristic reactions which interest us here only in so far as they are accompanied by the production of abnormal proteins. The question arises whether some of these abnormal proteins are produced by an interaction between the foreign protein and the normal proteolytic system of the organism in a manner which is comparable to the aforementioned cosubstrate action. One might consider, for instance, the possibility that the infection of a host by a virus protein might be explained as an alteration of the normal course of the coupled proteolytic and proteosynthetic reactions of the host tissues resulting, in the final outcome, in the synthetic production of more virus protein.

As a result of the latter part of this discussion, we recognize that proteolytic enzymes may act in two different manners. One type of reaction is represented by the specific action of a single enzyme upon a single pep-

peptide bond, while the other type of reaction consists in a sequence of coupled reactions involving several peptide bonds and several enzymes. If the assumption that the biological synthesis of proteins is effected by proteolytic enzymes⁵ is correct, then the second type of reaction must play a paramount role in protein synthesis. The proteins generated by living cells under normal or pathogenic conditions must be the product of coupled reactions in which many proteolytic enzymes and many substrates participate. The synergy of proteolytic enzymes in a sequence of coupled reactions requires a certain harmony between the specificities of the synergetic enzymes. A close study of the specificity requirements of the coupled reactions produced by proteolytic enzymes may help, in the future, to approach a better understanding of the biological synthesis of the many peptide bonds of a genuine protein.

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⁵ It has been suggested that enzymatic reactions other than proteolytic phenomena might also participate in the biological synthesis and degradation of proteins, as, for instance, oxidation-reduction phenomena (24).

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THE ENZYMATIC PROPERTIES OF PEPTIDASES

By

MARVIN J. JOHNSON AND JULIUS BERGER

Madison, Wis., and New London, Conn.

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I. Introduction

The term protease is usually employed as a general designation for any enzyme capable of hydrolyzing peptide linkages. Two sharply differentiated classes of proteases exist, proteinases and peptidases. Peptidases may split off single amino acids from one end or the other of the peptide chain, but only linkages adjacent to the end of the chain are split. The linkages hydrolyzed by proteinases, on the other hand, may have any posi-

tion in the chain. A proteinase, therefore, hydrolyzes a protein molecule into a number of polypeptide molecules. If these polypeptide molecules contain linkages attackable by the proteinase, they may be hydrolyzed to polypeptides of lower molecular weight. Even amino acids may be set free. The polypeptides resulting from proteinase action are attackable by peptidases. Naturally occurring peptidase systems are composed of a number of peptidases of varying specificity. It is with the properties of these individual peptidases that the present report is primarily concerned.

Among the properties commonly studied to differentiate and define peptidases have been the following: (a) The ability to hydrolyze dipeptides or tripeptides or both, and the relative rates at which these are split; (b) the ability to split the peptide linkage adjacent to the amino end or the carboxyl end of a substrate; (c) the necessity of a free amino or free carboxyl group or both on the peptide which is hydrolyzed; (d) the necessity of a hydrogen atom on the peptide nitrogen (--CO.NH--) of the substrate; and (e) the existence of specific activators such as metal ions and reducing agents.

In 1926, von Euler and Josephson (1), studying glycylglycine hydrolysis by erepsin, found that acylation of the free amino group of the peptide prevented hydrolysis. They also found that glycine inhibited the enzyme, but that aceturic acid did not. They concluded that a free amino group was necessary to permit combination of the enzyme with its substrate. In a second paper (2) they postulated that the hydrolysis of polypeptides by erepsin takes place by a combination of the enzyme with the amino group of the substrate, followed by splitting of the peptide linkage adjacent to the amino group. Thus two fundamental concepts were introduced: (a) that a peptidase has a point of attachment to its substrate apart from the linkage to be split, and (b) that a peptidase whose point of attachment is the terminal amino group of a polypeptide splits the peptide linkage adjacent to this group.

In 1928 Grassmann and Dyckerhoff (3) demonstrated that the peptidase of yeast autolysate could be resolved into dipeptide-splitting and polypeptide-splitting components. These components they called, respectively, dipeptidase and polypeptidase. Since the dipeptidase would not hydrolyze acylated dipeptides, they concluded that it required a free amino group. Since it hydrolyzed neither polypeptides nor amino acid amides, they concluded that dipeptidase also required in its substrate a carboxyl group adjacent to the linkage to be split.

The yeast polypeptidase, like the dipeptidase, did not hydrolyze acylated peptides. It was therefore postulated, without further evidence,

that it also required an intact amino group. It hydrolyzed polypeptide amides and esters, and therefore did not require a free carboxyl group. Since it split dipeptide and amino acid amides and dipeptide esters, but not free dipeptides, it was concluded that the proximity of a free carboxyl group to the linkage to be split inhibited the enzyme.

In 1929 Waldschmidt-Leitz, Balls and Waldschmidt-Graser (4) showed that hog erepsin, too, contained a dipeptide-splitting and a polypeptide-splitting component. In the same year, Waldschmidt-Leitz and Purr (5) found in pancreas extracts a peptidase which did not require a free amino group, since it hydrolyzed acylated peptides. A free carboxyl group, however, seemed to be necessary. This enzyme was called carboxypolypeptidase to differentiate it from the polypeptidase of intestine, which was named aminopolypeptidase.

The investigations referred to above, together with later papers containing substantiating evidence, constitute the groundwork of our present knowledge of peptidases. The demonstration that "erepsins" were not enzymatically pure, but consisted of mixtures of individual peptidases, and that these peptidases could be characterized as aminopeptidases or carboxypeptidases and as dipeptidases or polypeptidases, was sufficiently conclusive to find immediate acceptance by most workers in the field of proteolytic enzymes. The resulting stimulation of peptidase research during the next decade has resulted in our present relatively detailed knowledge of some peptidase systems.

1. *Criteria of Enzymatic Homogeneity*

In the early work on proteolytic enzymes a preparation was considered enzymatically pure when it was shown to be free from other *known* proteolytic enzymes. Such terms as "enzymatically homogeneous" and "proteolytically homogeneous" were applied to preparations from pancreas, yeast or intestine which were believed to contain only "erepsin" or "trypsin." When further work resulted in the production of an enzyme solution which hydrolyzed fewer substrates than its parent preparation, it, in turn, was immediately characterized as "homogeneous." Thus, intestinal aminopolypeptidase was assumed to be a single enzyme responsible for all the polypeptide-splitting properties of erepsin. Any property possessed by crude erepsin and not by aminopolypeptidase preparations was ascribed to dipeptidase, which was also tacitly assumed to be a single enzyme. The burden of proof, it was thought, definitely lay on the worker

who doubted the enzymatic homogeneity of a preparation, rather than on the one who postulated it.

As knowledge of peptidase action progressed, it became evident that inhomogeneity was much easier to demonstrate than enzymatic purity. It was found that different "dipeptidase" or "polypeptidase" samples varied greatly in specificity. It began to be apparent that enzymatic homogeneity was easy to assume, but often difficult to achieve. Unfortunately, however, most of the early work (and much of the later work) on peptidase specificity was done on preparations of doubtful homogeneity. Many of the resulting misconceptions are still being perpetuated in textbooks and reviews.

Almost all studies of enzymatic homogeneity are based on determinations of activity ratios. Suppose that enzyme preparation I attacks substrate A fifty times as rapidly as substrate B ($A:B = 50:1$). If preparation I is now subjected to some fractionation procedure and divided into two fractions, II and III, and if fraction II then possesses an activity ratio $A:B = 2:1$, and fraction III a ratio $A:B = 500:1$, it is difficult to escape the conclusion that preparation I contained at least two enzymes. On the other hand, if both fraction II and fraction III had shown ratios in the neighborhood of $50:1$, a conclusion that the original preparation was homogeneous would be suggested, but not necessarily justified, since the fractionation method used might very easily have failed to bring about a measurable separation. If, however, many different methods of fractionation (or fractional inactivation) all fail to change the activity ratio, it becomes probable that the preparation is homogeneous with regard to substrates A and B; that is, that it contains only one enzyme capable of attacking A or B.

When highly purified preparations can be obtained, another method of demonstrating enzymatic homogeneity becomes available. If it can be shown by the methods of protein chemistry that only one molecular species is present, enzymatic homogeneity can be assumed as a matter of course.

II. Polypeptidases

1. Carboxypolypeptidases

A chloroacetyltyrosine-splitting enzyme first recognized in pancreas extracts (5) was called carboxypolypeptidase. This enzyme hydrolyzed chloroacetyltyrosine most rapidly at pH 7.4. The hydrolysis by pancreas extracts of a large number of peptides, acyl peptides and acyl amino acids (6, 7, 8) was attributed to this peptidase.

Since the enzyme split acylated peptides, it did not require an amino group. *dl*-Leucylglycyl-*L*-tyrosine was split into *dl*-leucylglycine and *L*-tyrosine (9), showing that the enzyme split the linkage adjacent to the carboxyl group, and that it was able to split peptides containing unnatural amino acid residues when these were not adjacent to the linkage to be split. Bergmann, Zervas and Schleich (10) showed that pancreas extracts split chloroacetyltyrosine but not chloroacetyl-*N*-methyltyrosine. They concluded that carboxypolypeptidase required a hydrogen atom on the peptide nitrogen of its substrate.

In none of the above work was the homogeneity of "carboxypolypeptidase" investigated. On the other hand, Abderhalden and Schwab (11) have presented excellent evidence that carboxypolypeptidase is a mixture of peptidases. By a study of activity ratios, they were able to demonstrate the presence in "erepsin-free trypsin" (pancreas extracts not able to hydrolyze leucylglycine) of at least three peptidases. One of these, to which they gave the name "acylase," hydrolyzed halogen-acylated amino acids such as chloroacetyltyrosine and chloroacetylleucine. A second enzyme, called by them "tyrosine polypeptidase" hydrolyzed peptides such as leucyltyrosine, glycyltyrosine and leucylglycyltyrosine. A third, "leucine polypeptidase," hydrolyzed peptides containing leucine in the carboxyl position in the peptide chain. Waldschmidt-Leitz (6, 12) believed leucylglycyltyrosine splitting by pancreas preparations to be accomplished by the same enzyme which is responsible for chloroacetyltyrosine splitting. However, Abderhalden and Schwab were able to vary the chloroacetyltyrosine : leucylglycyltyrosine activity ratio of their preparations by a factor of fifty (from about 5 to about 0.1).

Abderhalden and Zeisset (13) have studied the splitting of optically active peptides by preparations from intestine (erepsin) and from pancreas (trypsin). They investigated optically active tripeptides of the type *A*-glycine-*C*, where the amino acid residues *A* and *C* were leucine, isoleucine or norleucine. Twelve tripeptides, containing the three leucines in various combinations, were investigated. It was found that all peptides in which *C* had the *L*-configuration were split by "trypsin" regardless of the configuration of *A*. With "erepsin" the opposite was true; all peptides in which *A* had the *L*-configuration were split, regardless of the configuration of *C*. "Trypsin" preparations did not attack any dipeptides of the *A*-glycine and glycine-*C* types. These results are strong supporting evidence for the presence in pancreas of a carboxypolypeptidase capable of hydrolyzing tripeptides having a leucine residue in carboxyl position. This enzyme cannot be identical with the chloroacetyltyrosine-hydrolyzing enzyme,

since pancreas preparations may be obtained (11) which vary in leucylglycylleucine : chloroacetyltyrosine activity ratios from 1.0 to approximately 100.

Although the number of carboxypeptidases in pancreatic extracts is unknown, one of them has been well characterized both enzymatically and chemically. Anson (14) has isolated a crystalline carboxypeptidase which attacks chloroacetyltyrosine rapidly. The enzyme was prepared from frozen beef pancreas. Since it is a pure crystalline protein, there seems to be no doubt regarding its enzymatic homogeneity. This enzyme appears to be identical with the chloroacetyltyrosine-splitting carboxypolypeptidase of Waldschmidt-Leitz. The crystalline enzyme was active even in the presence of formaldehyde, which presumably combines with the free amino groups of both enzyme and substrate. Fresh pancreas was found to contain, not active carboxypeptidase, but an inactive precursor, procarboxypeptidase. Partially purified preparations of this have been made by Anson (15). Solutions of the impure precursor are slowly activated on standing at 37°, but the activation is enormously hastened by the addition of trypsin, which appears to be a specific activator for procarboxypeptidase.

Abderhalden and Abderhalden (16) were unable to obtain preparations of procarboxypeptidase from pancreas. They have reported that by fractional inactivation by heat or formalin, the presence of a plurality of acylases could be demonstrated in a carboxypeptidase preparation. For this reason they suggest as specific substrates for carboxypeptidases polypeptides in which the amino group is blocked, as in benzoylleucylglycylleucine and β -naphthalenesulfonylglycyltyrosine. Their work has been criticized by Hofmann and Bergmann (17) who point out that if the initial enzyme concentrations are properly chosen so that the observed hydrolysis can be taken as a true expression of the quantity of active enzyme, no enzymatic inhomogeneity of crystalline carboxypeptidase is found.

Specificity studies on Anson's enzyme have been made by Bergmann and Fruton (18) and Hofmann and Bergmann (17). They found (18) that the crystalline enzyme did not hydrolyze leucylglycine or leucyldiglycine. While carbobenzoxy-glycyl-L-alanine was split very rapidly, the D-form was split extremely slowly.

Three times crystallized carboxypeptidase was found (17) to hydrolyze acylated dipeptides and acylated amino acids as shown in Table I. The acylated dipeptides, which were split at the linkage adjacent to the carboxyl group, were very rapidly hydrolyzed when the amino acid in carboxyl position was tyrosine or phenylalanine. Glycyltyrosine was slowly

split, but its acyl derivative carbobenzoxyglycyltyrosine was split 20,000 times more rapidly.

The necessity for the terminal carboxyl group was shown by the rapid hydrolysis of carbobenzoxy-glutamyl-*l*-phenylalanine and the resistance to

TABLE I
SPECIFICITY OF ANSON'S CRYSTALLINE CARBOXYPEPTIDASE

Calculations of activity ratios are made from data in the paper by Hofmann and Bergmann (17). The pH in all cases was 7.2 to 7.7. The temperature of incubation was 40°.

Substrate	Carboxy-peptidase (mg. N per cc.)	Incubation time (hours)	Hydrolysis (per cent)	Approximate relative activities
Carbobenzoxyglycyl- <i>l</i> -phenylalanine	0.00037	1	58	1,000
Carbobenzoxyglycyl- <i>l</i> -tyrosine	0.00037	1	37	640
Glycyl- <i>l</i> -tyrosine	0.645	4	11	0.03
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -phenylalanine	0.00037	1	13	220
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -phenylalanine amide	0.520	48*	5	0.002
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -tyrosine	0.00037	3	21	120
Carbobenzoxy- <i>l</i> -glycyl- <i>l</i> -alanine	0.083	1	66	5
<i>l</i> -Tyrosyl- <i>l</i> -tyrosine	0.520	1	†	1 (?)
α -Hippuryl- <i>l</i> -lysine	0.208	1	23	0.7
α -Hippuryl- ϵ -carbobenzoxy- <i>l</i> -lysine	0.520	24*	8	0.002
Carbobenzoxyglycyl- <i>l</i> -glutamic acid	0.510	24*	12	0.01
Carbobenzoxyglycylglycine	0.510	24*	30	0.03
Benzoylglycylglycine	0.520	5	64	0.2
Benzoyl- <i>l</i> -phenylalanine	0.520	24	20	0.02
Chloroacetyl- <i>l</i> -phenylalanine	0.0039	1*	25	86
Chloroacetyl- <i>l</i> -tryptophane	0.016	1	65	25
Chloroacetyl- <i>l</i> -tyrosine	0.00037	24	1	(0.7)†

* Incubation temperature 25°. The temperature coefficient is not taken into account in calculating activity ratios.

† From Table V of a previous paper (18), chloroacetyltyrosine appeared to be hydrolyzed about 4 times as rapidly as carbobenzoxyglycyl-*l*-alanine by crystalline carboxypeptidase.

‡ Tyrosine crystallization; the calculation of activity ratio assumes ca. 30 per cent hydrolysis.

hydrolysis of the amide of this compound. While the typical substrates did not contain any free amino group, certain substrates containing a free amino group, such as α -hippuryl-*l*-lysine, were nevertheless split. Contrary to Anson's findings (14), formaldehyde was found to inhibit carboxy-

peptidase action on three synthetic substrates. Unfortunately, Bergmann's papers (17, 18) give no significant data on the activity of the enzyme toward chloroacetyltyrosine. In (18) the amount of enzyme used is not indicated, while in (17) the hydrolysis obtained is too small to be significant. Although the only polypeptide investigated (leucyldiglycine) was not hydrolyzed, it may be assumed that the enzyme is capable of hydrolyzing polypeptides of suitable structure.

In summary, it may be concluded that the carboxypolypeptidase of Waldschmidt-Leitz is not an enzymatic entity, and that a number of carboxypeptidases are secreted by the animal pancreas, only one of which (Anson's enzyme) has been obtained free from other carboxypeptidases.

2. *Aminopeptidases*

Intestinal Aminopolypeptidase.—In 1929–33 Waldschmidt-Leitz, Balls and others (4, 9, 19, 20) found that after adsorption of hog intestinal mucosa extracts with Fe_2O_3 , a preparation remained which acted much more rapidly on polypeptides than on dipeptides. It was concluded that hog erepsin consisted of two enzymes, an aminopolypeptidase and a dipeptidase. Since aminopolypeptidase preparations did not hydrolyze acylated peptides, but did split peptide esters, it was concluded that a free amino group, but not a free carboxyl group, was required for hydrolysis. The peptide linkage split was found to be that adjacent to the free amino group. Balls and Köhler (19) purified the enzyme by acetone precipitation. They were able (20) to obtain a preparation which showed no change in activity ratios (4 peptides were used) on further adsorption. Intestinal aminopolypeptidase was found (9) to hydrolyze polypeptides, but not dipeptides or leucyldecarboxyglycine.

On the basis of inhibition experiments (21) it was concluded that besides the primary combination of the enzyme with the amino group of the substrate, a secondary combination with the nitrogen atom of the peptide linkage occurred.

Grassmann and his associates (22, 23) found that glycerol extracts of *fresh* intestine, but not of *dried* intestine, would split prolyldiglycine and prolylglycine rapidly. Since intestinal aminopolypeptidase was assumed to require an intact amino group in its substrate, and since dried preparations split leucyldiglycine but not prolyldiglycine, the splitting of prolyldiglycine by intestinal preparations was ascribed to "prolinase." Abderhalden and Nienburg (24) also found that extracts of *fresh* hog intestinal mucosa would split prolylglycine and prolyldiglycine rapidly and they

likewise attributed these hydrolyses to prolinase. Waldschmidt-Leitz (12) also considered aminopolypeptidase preparations made by Fe_2O_3 adsorption to contain prolinase.

TABLE II
SPECIFICITY COMPARISON OF PEPTIDASES

Substrate	Approximate relative rate of hydrolysis						
	Intestinal leucylpeptidase (35)*	Malt leucylpeptidase (35)	<i>Pseudomonas fluorescens</i> leucylpeptidase (36)	Intestinal aminopolypeptidase (25)	Yeast polypeptidase (Johnson (41))	Yeast polypeptidase (Grassmann (3, 39))	<i>Aspergillus parasiticus</i> polypeptidase (42)
<i>dl</i> -Leucyldiglycine	100	100	100	100	100	100	100
<i>dl</i> -Alanyldiglycine	25	10	10	1000	80	220	...
Triglycine	1.3	<1	1.4	600	3	40	<1
<i>dl</i> -Prolyldiglycine	1.3	1	2	700	<1
<i>dl</i> - <i>N</i> -Methylleucylglycine	0.7	<1	1.2	<1	0.0
<i>dl</i> - <i>N</i> -Methylalanyldiglycine	270
<i>dl</i> -Leucylmethylamine	15	<1	<1	3
<i>dl</i> -Leucylglycine	100	110	60	10	16	<1	3
<i>dl</i> -Alanylglycine	8	12	20	50	1
Diglycine	1.3	2	1	20	<1
<i>dl</i> -Prolylglycine	1.3	1	1	20
<i>dl</i> - <i>N</i> -Methylleucylglycine	1.1	<1	1
Sarcosyldiglycine	<1	<1	<1	110
Activators	Mg, Mn (33)	Mg, Mn (33)	Mg, Mn (33)	None found (33)	Zn, Cl^- (41)	None reported	Zn, reducing agents (33)

* The numbers in parentheses refer to the bibliography.

Later, Johnson (25) pointed out that the belief that aminopolypeptidase of hog erepsin required an intact amino group was based only on the fact that acylated peptides were not hydrolyzed. Substitution of the amino group in a manner that left its basicity unimpaired was shown not to pre-

vent hydrolysis. Thus, *N*-methylalanyldiglycine, sarcosyldiglycine and prolyldiglycine, although they lack an intact amino group, were rapidly split by an aminopolypeptidase preparation purified from *fresh* erepsin by acetone precipitation and Fe_2O_3 adsorption. It was shown that most of the leucyldiglycine-hydrolyzing activity of erepsin is *not* due to aminopolypeptidase (see section on leucylpeptidase), and that therefore the existence of peptidase preparations which split leucyldiglycine but not prolyldiglycine is not evidence for the inability of aminopolypeptidase to split prolyl peptides.

The relative activity of aminopolypeptidase toward a number of substrates was determined (25). It was found that dipeptides (alanylglycine, diglycine, prolylglycine, leucylglycine) were split at very appreciable rates (Table II). Alanylglycylmethylamine (decarboxylated alanyldiglycine) was split, showing that a carboxyl group was not essential. Monomethyl substitution of the amino group did not prevent hydrolysis, but *N*-dimethylalanyldiglycine was not split. The effect of various fractionation procedures on activity ratios was determined. It was found that the relative rates of hydrolysis of triglycine, *N*-methylalanyldiglycine, prolyldiglycine, alanylglycine and prolylglycine were not significantly changed by various adsorption, elution and precipitation procedures. The preparations used were thus indicated to be enzymatically pure with respect to the substrates studied.

Abderhalden and Abderhalden (26) have shown that extracts of dried intestinal mucosa split leucyldiglycine but not sarcosyldiglycine, and have questioned the conclusion (25) that aminopolypeptidase attacks the latter compound. As Gailey and Johnson (27) have pointed out, the leucyldiglycine activity of such dried preparations is due almost entirely to leucylpeptidase, which splits methylated peptides only very slowly.

Bergmann and Fruton (18) have found that none of the peptidases of intestinal mucosa extract are able to hydrolyze alanylsarcosylglycine. Since alanylglycylglycine is very rapidly split by aminopolypeptidase, this indicates that a hydrogen atom on the peptide nitrogen atom is essential for hydrolysis.

In conclusion, the properties of intestinal aminopolypeptidase may be summarized. It may be prepared from glycerol extract of intestinal mucosa in a state of apparent enzymatic homogeneity by a simple adsorption procedure (25). It hydrolyzes polypeptides more rapidly than dipeptides (25, 28). It does not hydrolyze acylated peptides (4), but does hydrolyze *N*-methylated peptides and prolyl peptides. It requires a hydrogen atom on the peptide nitrogen atom (18) but does not require a carboxyl group.

It splits the peptide linkage adjacent to the amino end of the polypeptide molecule. It is most active at pH 8 (29). In Table II the relative activity of aminopolypeptidase toward various peptides is compared with the activity of other peptidases.

Leucylpeptidase.—In 1929, Linderstrøm-Lang (30, 31, 32) showed the presence in erepsin of an enzyme, distinct from intestinal aminopolypeptidase and from the dipeptidase of Waldschmidt-Leitz, which hydrolyzed leucylglycine and leucyldiglycine rapidly. Johnson, Johnson and Peterson (29) found that this enzyme could very easily be prepared, free from aminopolypeptidase and dipeptidases, by acetone precipitation. It was found to be activated by magnesium ions. Berger and Johnson (33) later showed that manganese was a better activator, and that of a large number of ions tested, only magnesium and manganese had an activating effect.

Smith and Bergmann (34) have confirmed the manganese activation of leucylpeptidase from hog erepsin. The combination of the purified enzyme protein with the metal activator was found to be a time reaction. Like Linderstrøm-Lang (30–32), and Johnson, *et al.* (29), these authors found that most of the alanylglycine- and diglycine-splitting activity of erepsin was not due to leucylpeptidase.

Leucylpeptidase has been found (29, 35, 36) to hydrolyze leucyl peptides more rapidly than any others tested (Table II). It hydrolyzes leucyldiglycine and leucylglycine at approximately equal rates. It hydrolyzes leucylmethylamine and *N*-methylleucylglycine, showing that neither a carboxyl group nor an intact amino group is essential for hydrolysis. It should be pointed out, however, that substrates lacking an intact amino group are hydrolyzed only very slowly by leucylpeptidase, but relatively rapidly by aminopolypeptidase. For aminopolypeptidase, the most rapidly split substrate known is alanyldiglycine. Methylation of this peptide reduces the speed of hydrolysis to 25 per cent of its former value. Methylation of leucyldiglycine reduces the rate at which this peptide is hydrolyzed by leucylpeptidase to 0.5 per cent of its former value. Furthermore, prolyldiglycine is hydrolyzed very rapidly by aminopolypeptidase, but only very slowly by leucylpeptidase. Leucylpeptidase is widely distributed. It is produced by a variety of plants, animals, and bacteria (35, 36, 37, 38). Molds and yeast, however, apparently do not produce the enzyme (36).

Leucylpeptidase preparations can usually be obtained from crude extracts by a simple acetone precipitation. Such preparations, whether of plant, animal or bacterial origin, have typical leucylpeptidase properties

(Table II). Leucyldiglycine and leucylglycine are hydrolyzed rapidly and at approximately equal rates. Alanyl peptides are hydrolyzed more slowly, and glycyl peptides very slowly. The hydrolysis of all substrates is activated by magnesium and manganese.

The enzymatic homogeneity of leucylpeptidase has not been demonstrated. However, the fact that preparations exhibiting similar activity ratios and similar activation behavior may be obtained from a variety of sources indicates the wide distribution of an enzyme or group of enzymes having the characteristics associated with the term leucylpeptidase.

Yeast Polypeptidases.—Grassmann and co-workers (3, 39) obtained from yeast autolysates peptidase preparations which hydrolyzed a large number of polypeptides but no dipeptides. Dipeptide amides and esters, however, were split. Prolyldiglycine was attacked very slowly if at all (23). The enzyme was most active at pH 7.2. The enzymatic homogeneity of the preparations used in obtaining the specificity data was not investigated. Grassmann, Embden and Schneller (40) have obtained an extremely active preparation of the enzyme.

Johnson (41) has reported the isolation of a yeast polypeptidase protein. Although samples of this protein appear homogeneous in sedimentation and electrophoresis measurements, the variations encountered in specific activity show the presence of inactive material. There seems to be no reason for doubting the enzymatic homogeneity of the enzyme. This polypeptidase also hydrolyzes dipeptides, but much more slowly than tripeptides (Table II). It splits leucylmethyamine, showing that a carboxyl group is not essential. Prolyldiglycine is split extremely slowly. Leucyl peptides are split much more rapidly than corresponding glycyl peptides.

Zinc ions are apparently necessary for the activity of the enzyme. Zinc cannot be replaced by manganese, magnesium and other metals, with the possible exception of cobalt, which gives a definite and reproducible, though relatively small activation. Maximal activity is attained in the presence of 0.0001 *M* zinc ions. The presence of halide ions is also essential to the activity of the enzyme, although nitrate is able to replace halide to a certain extent. The enzyme splits leucyldiglycine most rapidly at pH 7.8, and alanyldiglycine most rapidly at pH 7.2. The enzyme differs in specificity from Grassmann's yeast polypeptidase but resembles (Table II) certain mold polypeptidases (42, 33) which are also zinc activated.

Other Amino-peptidases.—Many types of aminopeptidases other than those already described are known to exist. Since none of them has been studied in a state approach-

ing enzymatic homogeneity, they will be discussed in this review in the sections dealing with the organisms which produce them.

III. Dipeptidases

1. *Yeast Dipeptidase*

Grassmann and his collaborators (39, 43, 44) reported in 1927-28 the separation from yeast autolysate of a preparation which hydrolyzed dipeptides but not polypeptides. They concluded that the dipeptide-splitting activity was due to a single enzyme, which they called yeast dipeptidase. Specificity studies led to the conclusion (45) that yeast dipeptidase requires for its action the presence of both a free amino group and a free carboxyl group adjacent to the linkage to be split. This requirement is met only by dipeptides. Schneider (46) has found, however, that yeast dipeptidase preparations split *l*-alanylaminomalonyldiamide, which has no free carboxyl group.

Yeast dipeptidase was found to split alanylglycine very rapidly, leucylglycine rapidly and diglycine slowly. No investigation of its enzymatic homogeneity has been made. Grassmann has expressed the opinion (47) that variations in activity ratios among the dipeptidases are due to the presence of varying quantities of inhibiting substances, and that in all probability only one yeast dipeptidase exists. He shows (47, 48) that yeast dipeptidase has a high affinity for leucylglycine ($K_s = 0.001$) and a much lower one ($K_s = 0.117$) for diglycine. Thus amino acids and other competitive inhibitors affect diglycine splitting much more than leucylglycine splitting.

A number of observations have been made regarding activation of yeast dipeptidase. In 1926 von Euler and Josephson (49) reported that diglycine splitting by dialyzed yeast preparations was activated by addition of the dialysate. Grassmann and co-workers (50, 51) found that highly purified yeast aminopolypeptidase acquired the ability to split leucylglycine on addition of halide ions. Further activation was obtained upon addition of yeast kochsaft. Yeast dipeptidase preparations could be freed by adsorption from an activating substance replaceable by kochsaft. The halide activation of leucylglycine splitting by Grassmann's yeast aminopolypeptidase indicates that the aminopolypeptidase preparation used consisted in part of the yeast polypeptidase described by Johnson (41). Leucylglycine hydrolysis by this enzyme is activated by halide ions. The kochsaft activations may probably be ascribed to metals and reducing agents, which have been found by Berger and Johnson (33, 52) to activate

dipeptide hydrolysis by yeast enzymes. Grassmann's conclusion that he had separated yeast dipeptidase into coenzyme and apoenzyme fractions appears to require substantiation.

According to Grassmann, *et al.* (22), solutions of yeast dipeptidase split prolylglycine, but preparations which had been dried did not. The conclusion of the authors was that the yeast dipeptidase did not split prolylglycine but that some preparations were contaminated with "prolinase." It seems possible that the prolylglycine-hydrolyzing activity of yeast preparations may be due to a dipeptidase, which is not specific for prolyl peptides, just as most of the prolyldiglycine-hydrolyzing activity of intestinal erepsin has been shown to be due to aminopolypeptidase (25). Berger and Johnson (52) have shown that while prolylglycine hydrolysis by yeast enzymes, like diglycine and leucylglycine hydrolysis, is greatly activated by manganese ions and cysteine, alanyl-glycine hydrolysis is inhibited. Such data suggest the presence of several dipeptidases in yeast and the identity of yeast "prolinase" with one of these.

2. Intestinal Dipeptidase

Waldschmidt-Leitz, Balls and Waldschmidt-Graser (4) reported in 1929 that intestinal erepsin consisted of a polypeptidase and a dipeptidase. Balls and Köhler (28) were able to obtain by hematite adsorption and elution a dipeptidase preparation which had relatively little action on polypeptides, but which split dipeptides rapidly. Because crude erepsin splits glycyl-*p*-nitroaniline and similar compounds (28) as well as decarboxylated dipeptides (53), it was concluded that intestinal dipeptidase did not require a free carboxyl group in its substrate. The conclusion rested on the unsupported assumption that these compounds were split by the enzyme responsible for dipeptide hydrolysis. The necessity for an amino group in the substrate was assumed because of the resistance to hydrolysis of acylated peptides (7). The second point of attachment of the enzyme was considered to be the imino group. The assumption was tacitly made that only one dipeptidase was present in erepsin.

Linderstrøm-Lang (30-32) and Johnson, Johnson and Peterson (29) showed that hog erepsin contained a leucylpeptidase responsible for a large part of its leucylglycine-splitting activity. Yet this peptide is the most usual substrate used in the determination of "dipeptidase" activity. Intestinal aminopolypeptidase (25) also splits some dipeptides at a very appreciable rate. Crude erepsin, however, splits such dipeptides as alanyl-glycine and diglycine at a rate far exceeding that attributable to its

content of leucylpeptidase and aminopolypeptidase. The number of enzymes concerned in this dipeptidase activity is unknown.

Gailey and Johnson (27) investigated the activation of erepsin dipeptidases by metals and reducing agents. They reached the tentative conclusion that three of the dipeptidases could be identified as (a) an enzyme, splitting alanylglycine rapidly, for which no activator was found; (b) an enzyme hydrolyzing diglycine, greatly activated by cobalt; and (c) a manganese-activated enzyme (not leucylpeptidase) capable of hydrolyzing substrates lacking a free amino group of *l*-configuration, such as prolylglycine, *N*-methylleucylglycine and *d*-leucylglycine. Since separation experiments met with very little success, it was possible to reach only provisional conclusions. Variations in specificity and activation behavior, however, made it obvious that erepsin contained a number of dipeptidases.

Grassmann, *et al.* (22) found that dried erepsin preparations split leucylglycine but not prolylglycine, whereas fresh solutions split both substrates. They, therefore, postulated a "prolinase" distinct from "dipeptidase." Since dried erepsin preparations are rich in leucylpeptidase, the results of Grassmann, as has been pointed out elsewhere (27), show only that leucylpeptidase does not split prolylglycine rapidly. Whether prolyl peptides are split by a number of the intestinal dipeptidases or by only one of them is unknown. Prolylglycine splitting by crude erepsin, like diglycine splitting, is activated by metals and reducing agents (27).

IV. Antipodal Specificity of Peptidases

In general, it has been found that aminopeptidases hydrolyze peptides only if the amino acid bearing the free amino group of the peptide has the *l*-configuration. Carboxypeptidases have been found to require that the amino acid bearing the carboxyl group have the *l*-configuration (13). Bergmann and his associates (18, 54) have suggested an explanation for the optical specificity of peptidases. Since peptidases are believed to combine with the substrate at two points (the peptide nitrogen atom and either the amino or the carboxyl group), it is suggested that if the carbon atom adjacent to the peptide linkage has the *d*-configuration, such combination is difficult or impossible. For example, the α -carbon atom of the leucyl residue in a leucylglycine molecule is combined with a carbonyl group, an amino group, an isobutyl group and a hydrogen atom. If the carbon atom has the *d*-configuration, the large isobutyl group may project in a manner to prevent the combination of the enzyme with the amino group and peptide nitrogen. If the carbon atom has the *l*-configuration,

the positions of the isobutyl group and hydrogen atom are interchanged, and the small hydrogen atom offers no steric hindrance. In the case of dipeptides it is postulated that the enzyme combines with both the amino and carboxyl groups. It is pointed out that *d*-alanyl peptides, in which the inhibiting group is the relatively small methyl group, should be hydrolyzed to some extent. Such is found to be the case, but it is not demonstrated that the *d*-alanyl peptides are split more rapidly than those *d*-peptides in which the inhibiting group is larger.

Berger, Johnson and Baumann (55) have shown that in the presence of metal activators, the enzymes of yeast and intestine split *d*-leucylglycine at an appreciable rate, and that peptidase preparations from *Bacillus megatherium* hydrolyze this dipeptide almost as rapidly as its *l*-enantiomorph. Preparations from *Leuconostoc mesenteroides* and *Clostridium butylicum* hydrolyzed *d*-leucyldiglycine one-fifth to one-half as rapidly as *dl*-leucyldiglycine.

It is interesting to note that regardless of the source of the enzyme, hydrolysis of *d*-peptides was always activated by manganous salts and cysteine and that cobalt ions in several cases were also strongly activating. Gailey and Johnson (27) found that in some intestinal preparations, *d*-leucylglycine was hydrolyzed almost as rapidly as the *dl*-form.

V. Peptidase Systems

1. Intestinal Mucosa

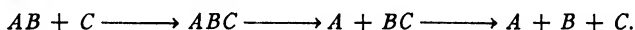
Animal intestinal mucosa undoubtedly contains many peptidases other than those already characterized. Such techniques as the use of test substrates other than the very simple peptides usually employed, the further investigation of the hydrolysis of *d*-peptides, the development of new preparative methods and the qualitative comparison of the intestinal peptidase complexes of different animals should result in much new knowledge regarding peptidases. A number of leads have been obtained, such as the chloroacetyl-*o*-nitroaniline and benzoyltriglycine hydrolyzing enzymes of Balls and Köhler (56). (See also Abderhalden, *et al.* (57)). Bergmann and Fruton (18) showed that crude erepsin split alanyldiglycine rapidly but alanylsarcosylglycine not at all. It was concluded that the aminopeptidases of erepsin require a peptide hydrogen (a hydrogen atom on the peptide nitrogen atom) for their action. However, erepsin did split glycylproline (18, 58) which lacks a peptide hydrogen. Therefore, they postulated the existence of a new enzyme for which the name "prolidase" was proposed. Glycylsarcosine, similar to glycylproline in its lack of a peptide

hydrogen, was not split by the prolidase. The identity of prolidase with one of the dipeptidases (27) of crepsin was by no means excluded, since only leucylglycine was used as an indicator of dipeptidase activity.

2. *Pancreas*

The pancreatic proteinases do not come within the scope of this review. Of the peptidases secreted by the pancreas, only a carboxypeptidase has been well characterized. The presence of other peptidases has been discussed by Abderhalden and Schwab (11, 59).

Another pancreatic enzyme, "protaminase," has been studied by Waldschmidt-Leitz and Kofranyi (60). It does not attack proteins, but splits arginine from the carboxyl end of the elupecin molecule. It should therefore, perhaps, be classified as a carboxypeptidase. Abderhalden and von Ehrenwall (61, 62) have made the interesting observation that crude trypsin solutions, not possessing leucylglycine-hydrolyzing activity, develop such activity upon addition of any one of a number of amino acids. This phenomenon is especially interesting in view of the "cosubstrate effect" discovered by Behrens and Bergmann (63) who presented strong evidence that an analogous phenomenon exhibited by papain was due to preliminary enzymatic synthesis of the added activator with the substrate, followed by hydrolysis (at a different linkage) of the resulting compound. The process might be represented thus:



Tsuchiya (64) has reported that hydrolysis of alanylglycine, a typical dipeptidase substrate, by pancreas extracts is greatly activated by zinc ions.

3. *Other Animal Peptidases*

Although liver, kidney, spleen and other organs are known to be rich in peptidases, very little detailed work has been done on these enzymes. Fruton and Bergmann (65) have found that a partially purified beef spleen "cathepsin" preparation was capable of hydrolyzing simple dipeptides and tripeptides at pH 4.5. Reducing agents such as cysteine and ascorbic acid activated this hydrolysis. The pH optimum for hydrolysis of L-leucylglycine was 4.4, with cysteine present as activator. Waldschmidt-Leitz and others (66) have reported the presence in animal organs of a carboxypeptidase optimally active at acid pH values, and of other peptidases. Abderhalden and Schwab (67) have shown that liver press juice hydrolyzes a variety of peptides.

The peptidases of animal blood have been studied by Grassmann and Heyde (68), Abderhalden and Hanson (69) and Weil and Russel (70). Animal blood plasma (or serum) hydrolyzes both polypeptides and dipeptides. Chloroacetyltyrosine and prolylglycine are not attacked (68). Berger, *et al.* (55), reported leucylglycine hydrolysis by rat and human sera to be activated by manganese ions, and Maschmann (38, 71) found serum peptidases to be activated by Mg, Mn and Co ions. The claim of Waldschmidt-Leitz and Mayer (72) that human cancer sera contain *d*-peptidases has led to a series of papers on *d*-peptidases (55, 73-82) from which the general conclusion appears to be that both normal and cancer sera may hydrolyze certain *d*-peptides extremely slowly and with considerable irregularity.

4. *Peptidases of Higher Plants*

Very little is known concerning the constitution of plant peptidase systems. Ambros and Harteneck (83) found dipeptidase activity in extracts from a large number of plants. Linderstrøm-Lang and Holter (84) studied the distribution of alanylglycine and leucylglycine-hydrolyzing activity in barley root tips and cotyledons. Linderstrøm-Lang and Sato (31) and Sato (85) found barley malt to contain a leucylpeptidase in addition to other peptidases. Berger and Johnson (35) have reported the presence in extracts of malt, cabbage and spinach of a manganese-activated leucylpeptidase.

5. *Peptidases of Fungi*

The known peptidases of yeast have already been discussed. Although molds are in general rich in peptidase activity, they have received but little attention. Johnson and Peterson (42), who studied the peptidase system of *Aspergillus parasiticus*, concluded that at least four peptidases were present. One of these, apparently an aminopolypeptidase, was obtained in a partially purified state. No proofs of enzymatic homogeneity were given, however. This enzyme, which was later (33) found to be activated by zinc ions and reducing agents, resembles in many respects the pure yeast polypeptidase described by Johnson (41). Berger and Johnson (33) showed that polypeptide hydrolysis by a number of molds was activated by zinc ions and reducing agents. Berger, *et al.* (86) studied the hydrolysis of leucyldiglycine, leucylglycine, triglycine, diglycine and chloroacetyltyrosine by preparations from a large number of mold species. The activity ratios toward different substrates varied greatly with species. The

peptidase activity of the mycelium was much greater when it was grown on a skim milk medium than when grown on inorganic salts-glucose medium. Otani (87) studied acylase production by molds. Benzoylleucylglycine was readily decomposed.

6. Bacterial Peptidases

Although the peptidases of bacteria have received much attention during the past few years, in no case has a bacterial peptidase of known enzymatic purity been prepared. Thus far, work has been largely confined to orientation studies in the course of which a good deal of knowledge of the general properties of the peptidases of various bacterial types has accumulated. Many of the peptidase types found in other sources occur also in bacteria, but there appear to exist a number of peptidase types which are peculiarly bacterial. Leucylpeptidases have been found by Berger, *et al.* (36, 88), to be present in the cells of a number of aerobic bacteria. Bacterial leucylpeptidases appear to be very similar in enzymatic properties to leucylpeptidases from other sources. It is impossible, from present data, to determine whether bacteria contain peptidases similar to intestinal aminopolypeptidase, since this peptidase is apparently not characterized by the specific activation behavior which facilitates detection of some peptidases. Many crude bacterial peptidase preparations (88), however, show pH and specificity relations which are at least consistent with the presence of such an enzyme. Certain bacteria, for example, *Bacillus megatherium* (52) and *Phylomonas lumefaciens* (33), show an activation of dipeptide splitting by metals and reducing agents which is very similar to the corresponding activation behavior of the dipeptidases of intestine and yeast.

Bacterial peptidases, particularly those of anaerobic bacteria, very frequently are found to be activated by reducing agents, divalent metals or both. Berger, Johnson and Peterson (89) found that any one of a number of divalent metals (Zn, Pb, Cu, Mn, Sn, Cd, Hg) would, in concentrations ranging from 10^{-3} M to 10^{-5} M, greatly accelerate hydrolysis of alanyldiglycine by peptidases from *Leuconostoc mesenteroides*. Metal activation of hydrolysis of other peptides was also obtained. It was subsequently found (88) that peptide hydrolysis by peptidases of this organism was also activated by a number of reducing agents, such as cysteine, H_2S , HCN and *p*-methylaminophenol. It was found that peptidases of other anaerobes were also activated by reducing agents.

Maschmann, in a series of papers (90-96) has reported the peptidases of culture filtrates of a number of species of the genus *Clostridium* to be greatly activated by metals and reducing agents. Of all the metals tried, only Fe^{++} and Mn^{++} gave activation. A number of reducing agents were effective, but in general, Fe^{++} + cysteine gave optimal activation (93). Since the metal and the reducing agent together give more than additive activation, Maschmann postulates, without further evidence, that the

peptidase can exist in an oxidized form and a reduced form, and that only a metal complex of the reduced form is enzymatically active (93).

Because of the great variations Maschmann encountered in dipeptide activity ratios, he believes (92, 95) that several *Clostridium* dipeptidases exist, which are present in varying proportions in different preparations. He was also able, by ammonium sulfate fractionation, to obtain (95, 96) with *Cl. histolyticum* and *Cl. botulinum*, enormous (400-fold) changes in leucyldiglycine : triglycine activity ratios, showing at least two polypeptidases to be present. Both the leucyldiglycine enzyme and the triglycine enzyme were activated by Fe^{++} + cysteine.

Bacterial peptidase systems differ from those of other organisms in that they frequently contain peptidases whose optimum pH is in the acid range (pH 5 to 6). Gorbach (97) investigating the effect of pH on leucylglycine and leucyldiglycine hydrolysis by culture filtrates from the acidoproteolytes of Gorini, found two pH optima, one varying from pH 7 to pH 8.4 with different species, and another at pH 4.8. These "acid peptidases" were believed to be characteristic of acidoproteolytic organisms. Berger, Johnson and Peterson (89) in an investigation of the intracellular peptidases of *Leuconostoc mesenteroides* found acid peptidases to be present in this organism. The acid peptidases were not activated by divalent metals, whereas the peptidases active at pH 8 did show metal activation. The presence of acid peptidases in *Propionibacterium* and *Lactobacillus* species was also reported (88).

Many bacteria contain peptidases capable of rapid hydrolysis of *d*-peptides. Berger, Johnson and Baumann (55) have studied the relative rates at which *d*- and *l*-peptides were split by peptidase preparations from animal, plant and bacterial sources. It was found that the unnatural forms of leucylglycine and leucyldiglycine were hydrolyzed slowly by most peptidase systems, but some bacterial preparations gave relatively rapid hydrolysis of the unnatural peptides, the hydrolysis rate in some cases approaching the rate at which the natural peptide was split.

The secretion of peptidases by the bacterial cell has been studied but little. In no case has it been established that living cells secrete peptidases into the culture medium. Elberg and Meyer (98), for example, working with *Cl. parabotulinum* find that peptidases appeared in the medium only after 24 hours' growth, and that the peptidase content of the culture medium rose greatly as cell autolysis took place. Kocholaty, Smith and Weil (99) report that peptidases are liberated by *Cl. histolyticum* cells only when autolysis takes place. Berger, *et al.* (88) found that in 24-hour cultures of *B. megatherium*, the medium contained more peptidase activity than the cells, whereas in a 45-hour *Escherichia coli* culture, most of the peptidases remained in the cells.

VI. Conclusion

Present knowledge of peptidase systems is still very meager. In hog erepsin, the peptidase complex about which most is known, an aminopolypeptidase and a leucylpeptidase have been characterized. The presence of a plurality of dipeptidases is realized, but their separation and characterization await the development of improved preparative methods. In brewer's yeast, one polypeptidase has been isolated as a protein, and another polypeptidase and a dipeptidase (or dipeptidase complex) have been partially characterized. The only peptidase which has thus far been crystallized is a carboxypeptidase from pancreas. Concerning other pancreatic peptidases very little is known. Investigation of other peptidase systems has been limited largely if not entirely to general studies on a whole peptidase complex.

It is unfortunate that too few current papers take sufficient cognizance of our complete ignorance of the enzymatic constitution of most peptidase systems. In many investigations the leucyldiglycine hydrolyzing activity of preparations from any source is attributed to "aminopolypeptidase," even in the absence of any evidence that splitting is taking place at the amino end of the chain.

In the same way, leucylglycine or alanylglycine hydrolysis is usually attributed to "dipeptidase." Apparently there has arisen a firmly rooted conception that, in all probability, any peptidase system consists of two components, an aminopolypeptidase, which may be unerringly detected by the use of leucyldiglycine as a substrate, and a dipeptidase, which can be tested for by the use of almost any dipeptide. In the most recent review on proteases, that of Bersin (100) "aminopeptidase" is treated as a single enzyme, occurring in a variety of materials. In the description of its specificity behavior, no distinction is made among leucyldiglycine-hydrolyzing enzymes from different sources. "Dipeptidase" is similarly treated as a single enzyme.

It has been the chief aim of this review to summarize existing knowledge of the enzymatic characteristics of certain peptidases, and to emphasize existing ignorance of the number and nature of the enzymes constituting most naturally occurring peptidase systems.

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DIAMIN-OXYDASE

Von

E. ALBERT ZELLER

Basel, Switzerland

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I. Natur und systematische Stellung der Diamin-oxydase

Im Jahre 1929 beschrieb Best (1) ein Histamin inaktivierendes Ferment, das von dem Entdecker und seinen Mitarbeitern eingehend studiert und als Histaminase bezeichnet worden ist (2, 3, 4). Neun Jahre später, 1938, wurde von Zeller ein Enzym, die Diamin-oxydase, gefunden, das Diamine wie Putrescin, Cadaverin und Agmatin abbaut (5). Bald darauf wurde die Identität der beiden Fermente nachgewiesen (6, 7). Fast gleichzeitig stellte auch Felix die enzymatische Angreifbarkeit der genannten Diamine fest (8).

Die Diamin-oxydase (DO) ist eine Dehydrase, bei der molekularer Sauerstoff als Wasserstoffacceptor dient. Dieser geht dabei in Wasserstoffperoxyd über (6). Sie gehört somit zur Gruppe der als Aero-dehydrasen resp. Oxy-hydrasen bezeichneten Fermente. Diese Gruppe umfasst offenbar mindestens 2 wesentlich von einander verschiedene Untergruppen von Enzymen, von denen die eine zu den Schwermetallfermenten gehört und von Blausäure gehemmt wird (Beispiel: Ascorbinsäure-oxydase) und die andere, u.a. Flavinfermente umfasst, die durch Blausäure nicht beeinflusst werden (Beispiel: *d*-Aminosäure-oxydase). Obwohl die DO durch Blausäure inaktiviert wird, gehört sie doch zu der 2. Untergruppe; bei der DO beruht die Hemmung durch Blausäure nicht auf deren Vermögen, Schwermetalle zu entionisieren, sondern mit Carbonylverbindungen Cyanhydrine zu bilden. Überdies konnte wahrscheinlich gemacht werden, dass am Aufbau der DO ein Flavin beteiligt ist (9). Die Einwirkung der *d*-Aminosäure-oxydase, der Monoamin-oxydase und der DO auf ihre Substrate kann durch ein und dieselbe Gleichung dargestellt werden. Diese Enzyme bilden offenbar innerhalb der 2. Untergruppe eine besondere Abteilung.

Die Bezeichnung Histaminase verwenden wir, obwohl sie die ältere ist, nicht mehr, weil der enzymatische Abbau von Histamin nur ein Sonderfall aus einer ganzen Reihe von enzymatischen Umwandlungen biologisch wichtiger Di- und Polyamine darstellt. In ähnlicher Weise wurde an Stelle derspeziellen Ausdrücke Amin-oxydase, Tyraminase und Adrenalin-oxydase der allgemeinere der Monoamin-oxydase gesetzt, als nachgewiesen worden war, dass es sich bei allen 3 Fermenten stets um dasselbe handelte (10, 11). Franke (12) nennt das Ferment nach der Oppenheimer'schen Nomenklatur Diamin-oxhydrase. Da aber, wie im voranstehenden Abschnitt angedeutet wurde, die Gruppe der Oxyhydrasen uneinheitlich ist, bleiben wir vorläufig bei der rein deskriptiven Bezeichnung der Diamin-oxydase.

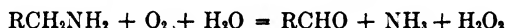
II. Messung der Diamin-oxydase

Die folgenden Reaktionsbedingungen haben sich für die Messung der DO bewährt: pH 6.8 bis 7.6, gegen Phosphatpuffer gründlich dialysierte Fermentlösungen, Sauerstoff- ev. Luftatmosphäre, Zusatz von Octylalkohol.

Von Bedeutung wegen der Beeinflussung der Reaktionsgeschwindigkeit ist die Grösse der Substratkonzentration. Beim Histamin und Agmatin findet sich ein sehr ausgeprägtes Konzentrationsoptimum (vgl. Kapitel VII). Bei vergleichenden Untersuchungen muss deshalb dieses Optimum

ermittelt und für die Messung eingehalten werden. Bei Cadaverin und Putrescin ist dieses Optimum nur schwach ausgeprägt, sodass die Bestimmung des Optimums gewöhnlich nicht nötig ist. Eine Ausnahme bilden Hemmungsversuche, bei denen der Summationseffekt auftritt (vgl. Kapitel VII).

Für die DO-Reaktion gilt folgende Gleichung (6, 9)



Als günstig für die Bestimmung der DO erwies sich die Messung (a) der noch vorhandenen Substratmenge, (b) des Sauerstoffverbrauchs, (c) des freigesetzten Ammoniaks und (d) des gebildeten Peroxyds.

(a) Da es sich beim Histamin um einen biologisch aktiven Stoff handelt, kann die Messung auf pharmakologischem Wege geschehen. Es werden 2 Methoden verwendet: Die Messung der Blutdruckabnahme der narkotisierten Katze (1) und die kymographische Registrierung der Kontraktion des isolierten Meerschweinchendarmes (13). Rein chemisch könnte die Substratkonzentration durch den nach Van Slyke bestimm- baren Aminostickstoff erfasst werden (4).

(b) Der Sauerstoffverbrauch kann, wenn es sich nicht um allzu kleine Fermentkonzentrationen handelt, in der Warburg'schen Apparatur manometrisch bestimmt werden (14, 6, 15). Das Verfahren ist auch auf diesem Gebiet der vielseitigsten Anwendung fähig.

(c) Für die Messung des gebildeten Ammoniaks wurden zuerst die *Van Slyke-Cullen'sche* (3) und die *Folin'sche* (13) Methode herangezogen, später die *Parnas'sche* Vakuumdestillation (6) und die *Conway'sche* Diffusionsanalyse (16), welche letztere auch auf die Manometergefäße der Warburg'schen Apparatur übertragen wurde (17). Die *Parnas'sche* und die *Conway'sche* Methode, bei denen das destillierte Ammoniak mit Nessler's Reagens und Stufenphotometer bestimmt wird, haben sich besonders bewährt, weil sie wegen der Kleinheit der noch erfassbaren Mengen unter Umständen schon in wenigen Minuten nach Beginn der Spaltung exakte Bestimmungen erlauben.

(d) Durch das sich bildende Peroxyd können verschiedene zugesetzte Substanzen, u.a. Indigodisulfonat oxydiert werden. Bei dieser Indigoentfärbung wird entweder die Entfärbungszeit oder die photometrisch nach einer bestimmten Zeit noch vorhandene Indigomenge ermittelt (18, 19, 20, 21).

Der Abbau von 10^{-6} Mol Cadaverin pro Stunde entspricht einer DO-Einheit. In 1 g. Nierenrinde des Schweines sind beispielsweise 30 DO-Einheiten, in 1 cm.³ Menschen- serum (von Nichtschwängern) 0.01, im Schwangerserum 0.05–0.2 DO-Einheiten enthalten.

Die Organe, in welchen die DO bestimmt werden soll, werden (nach eventueller Zerkleinerung im Latapie) mit Quarzsand und mit der drei- bis fünffachen Menge 2.5-proz. Kochsalzlösung fein verrieben. Die Suspension wird zentrifugiert oder durch ein Nesseltuch getrieben.

III. Gewinnung aktiver Diamin-oxydase-Präparate

Die Nierenrinde von Schweinen bildet ein günstiges Ausgangsmaterial für DO-Präparate. Aus frischer Niere lassen sich in der im vorangehenden

Kapitel angedeuteten Weise sehr aktive Lösungen gewinnen. Durch Behandlung von Nierenbrei mit eiskaltem Aceton stellen wir ein mehrere Monate haltbares Trockenpräparat her, aus dem durch Extraktion mit Kochsalzlösung die Fermentlösung hergestellt wird. Diese kann im Vakuum eingedampft werden, wobei ein wasserlösliches, von vielen Ballaststoffen befreites Pulver erhalten wird.

Die bisher unternommenen Versuche (3, 4, 13) zur Reinigung der DO mit Hilfe von Fällungen, Adsorptionen und Elutionen sind noch nicht allzu weit getrieben worden. Immerhin wurden Präparate erhalten, die das zehnfache der ursprünglichen "Histaminase"-Konzentration aufwiesen (3).

IV. Spezifität der Diamin-oxydase

Es sind durch DO-Präparate bisher folgende 13 Basen oxydativ desaminiert worden (5, 6, 18, 19, 22).

1. Aethylendiamin	$\text{H}_2\text{N}(\text{CH}_2)_2\text{NH}_2$
2. Trimethylendiamin	$\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}_2$
3. Putrescin	$\text{H}_2\text{N}(\text{CH}_2)_4\text{NH}_2$
4. Cadaverin	$\text{H}_2\text{N}(\text{CH}_2)_5\text{NH}_2$
5. Spermidin	$\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$
6.)	$\left\{ \begin{array}{l} \text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_5\text{NH}_2 \\ \text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_6\text{NH}_2 \\ \text{NH}_2(\text{CH}_2)_4\text{NH}(\text{CH}_2)_4\text{NH}_2 \\ \text{NH}_2(\text{CH}_2)_4\text{NH}_4(\text{CH}_2)_5\text{NH}_2 \\ \text{NH}_2(\text{CH}_2)_5\text{NH}(\text{CH}_2)_5\text{NH}_2 \end{array} \right.$
7.)	
8.)	
9.)	
10.)	
11. Spermin	$\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$
12. Agmatin	$\text{NH}_2\text{C}(\text{NH})\text{NH}_2(\text{CH}_2)_4\text{NH}_2$
13. Histamin	$\text{N}-\text{C}-\text{CH}_2-\text{CH}_2-\text{NH}_2$
	$\begin{array}{c} \parallel \quad \parallel \\ \text{HC} \quad \text{CH} \\ \diagdown \quad \diagup \\ \text{NH} \end{array}$

Es lassen sich (z.B. aus Acetontrockenpulver) Fermentlösungen gewinnen, die all diese Körper abbauen, dagegen ausser Histamin keines der Imidazolderivate: Imidazolyl-aldehyd, -essigsäure, -propionsäure, -milchsäure, Histidin und Harnsäure, noch irgend ein Monoamin wie Amylamin, Tyramin, Tryptamin, Adrenalin, Phenyl-oxy-aethylamin und Ephedrin (2, 5, 6, 7, 14). Das Ferment besitzt demnach keine besondere Beziehung zum Imidazolring, noch zu einer einzelnen Aminogruppe.

Das Gemeinsame aller Substrate ist das Vorhandensein von mindestens

2 Aminogruppen, von denen die eine substituiert sein kann. Wenn an die Stelle beider Aminogruppen Guanidinreste treten, so werden diese Stoffe wie Arcain und Synthalin (Tetra- bzw. Dekamethylendiguanidin) wohl von der DO gebunden, aber nicht enzymatisch angegriffen. Das Diamin Aneurin (Vitamin B₁, Thiamin) (22) und das cyclische Diamin Piperazin (23) werden ebenfalls nur gebunden und nicht oxydiert.

Ferner greifen geeignete DO-Lösungen auch keine *d*-Aminosäuren (24), noch Diamin-monokarbonsäuren (6), Cholin (20) und Hypoxanthin an (24). Die DO ist somit mit Sicherheit gegenüber der *d*-Aminosäure-, Monoamin-, Cholin-, Xanthin- und Urico-oxydase abzugrenzen. Es existiert ein reiches experimentelles Material zur Unterstützung dieser Anschauung, doch sei im Folgenden nur die Frage der Identität der Histaminase und der DO etwas ausführlicher diskutiert.

In zahlreichen Konkurrenzversuchen zwischen einfachen Diaminen und Histamin wurde ausnahmslos eine competitive Hemmung gefunden. Das Ergebnis solcher Versuche ist aber nur im Zusammenhang mit andern absolut beweiskräftig, weil ähnliche Erscheinungen auftreten können, wenn 2 Fermente tatsächlich voneinander verschieden sind, aber eine gemeinsame Komponente besitzen, wie es für die Monoamin-oxydase und die Urico-oxydase gezeigt werden konnte (23).

In vielen Hunderten von Versuchen mit vielen Organen der verschiedensten Tierarten, die sogar verschiedenen Stämmen angehörten, wurde regelmässig festgestellt, dass zwischen dem Histamin- und dem Diaminabbau immer eine strenge Parallelität herrscht. Die Angaben über die Verteilung der Histaminase, die fast ausschliesslich mit biologischen Methoden gewonnen wurden, stimmen genau mit denen über die DO mit rein chemisch-physikalischen Methoden ermittelten DO überein. Eine auffallende Bestätigung für die Annahme der Identität bildete die Erfahrung, dass die Oxydation des Histamins und des Cadaverins durch das menschliche Serum während der Schwangerschaft in gleichem Ausmasse beschleunigt wird.

Es wurde sogar vermutet, dass die bei *Bacillus pyocyaneus* gefundene Histaminase ebenfalls eine DO sei, weil der Histaminabbau durch Putrescin competitiv gehemmt wurde (25).

Histaminase und DO verhalten sich gegenüber der Einwirkung der vielen Hemmungsstoffe völlig gleich. Wenn Unterschiede auftraten, so liessen sie sich eindeutig auf die von Substrat zu Substrat wechselnden Affinitäten zurückführen.

Zusammenfassend ist festzustellen, dass ein erhebliches experimentelles Material vorliegt, das für die Identität von Histaminase und DO spricht,

ohne dass ein einziges Ergebnis bekannt geworden wäre, das dieser Annahme widerspricht.

V. Affinität zwischen der Diamin-oxydase und ihren Substraten

Aus Konkurrenzversuchen ging hervor, dass die Affinitäten der verschiedenen Substrate der DO voneinander wesentlich abweichen (6, 18, 22). Die qualitativen Ergebnisse wurden durch die quantitativen Bestimmungen der Michaelis'schen Konstanten bestätigt. Unter den angegebenen Versuchsbedingungen fanden sich (22):

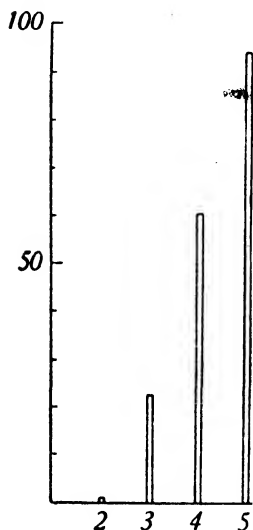


Fig. 1.—Abszisse: Zahl der C-Atome (2-5). Ordinate: Stickstoff in %.

	k_M	$1/k_M$
Putrescin	0.0012	840
Cadaverin	0.00056	1800
Histamin	0.00050	2000

Die Affinität ist von der Länge der Methylenkette abhängig. Sie nimmt vom Aethylendiamin bis zum Putrescin ab, um beim Cadaverin wieder anzusteigen. Diese Erkenntnis wurde hauptsächlich durch die Untersuchung der Abbaugeschwindigkeit der von v. Braun (26) dargestellten Spermidinhomologe gewonnen (22). Die Substrate der DO binden sich bei der Bildung des Ferment-Substratkomplexes mit 2 basischen Gruppen an das Enzym (Kapitel VIII). Triamine vom Typus des Spermidins können deshalb auf 2 verschiedene Arten mit der DO kombinieren, entweder mit der längeren oder kürzern Kette, und je nach dem wird die Aminogruppe der einen

oder andern aboxydiert. Welche Kette aber abgebaut wird, kann aus der Reaktionsgeschwindigkeit gesehen werden, da diese eine Funktion der Länge der Methylenkette ist und vom Aethylendiamin bis zum Cadaverin regelmässig zunimmt (18) (Fig. 1).*

Bei der Affinität kommt es aber nicht nur auf die gegenseitige Stellung, sondern auch auf die Konstitution der beiden basischen Gruppen an, sodass deren Affinität gesondert berücksichtigt werden muss. Aus Hemmungsversuchen mit Monoaminen, Guanidinen oder Imidazol (Glyoxalin) geht

* Die Figuren stammen alle aus Veröffentlichungen aus *Helvetica Chimie Acta*. Mit der freundlichen Erlaubnis der Redaktion werden sie hier wiedergegeben.

hervor, dass die Affinität der beiden letztern sehr viel grösser als die der Monoamine ist (20, 23, 27). Die Affinität der beiden basischen Gruppen des Histamins oder des Agmatins zu den entsprechenden Gruppen der Haftstelle der DO sind somit von einander sehr verschieden und bedingen das Auftreten des so ausgeprägten Optimums der Substratkonzentration (Kapitel VIII) bei diesen Diaminen.

Diese Überlegungen über die Affinität der DO zu ihren Substraten hat nicht nur ein rein fermentchemisches Interesse, sondern auch ein biologisches. Die Mehrzahl der aufgezählten Substrate kommen im tierischen Organismus vor, ebenso einige Hemmungsstoffe wie Aneurin und Guanidine. Es muss also zwischen diesen Stoffen zu einer Konkurrenz um die DO kommen, wobei neben den Konzentrationen der betreffenden Substanzen in erster Linie die Affinitäten ausschlaggebend sind. So hat beispielsweise Spermin eine grosse Affinität zur DO, wird aber verhältnismässig langsam abgebaut. Während dieser Zeit ist die DO für den Abbau anderer Diamine mit geringerer Affinität blockiert, sodass jene im Stoffwechsel sich anhäufen.

Möglicherweise bestehen auch Beziehungen zwischen der Grösse der Affinität und der Hemmung der biologischen Urochloralsynthese durch Diamine, da das Ausmass dieser Hemmung ein paralleles Verhalten zur Affinität erkennen lässt und wie diese beim Putrescin ein Minimum aufweist (28).

VI. Inhibitoren und Aktivatoren

Von organischen Stoffen wirken Ammoniumionen (2) schwach, Calciumionen (4) stärker und Fluoride (18) überhaupt nicht auf die DO hemmend ein. Urethan (6), Jodacetat, Kohlenoxyd (13) und Malonat (18) sind innerhalb der üblichen Konzentrationen ohne wesentlichen Einfluss.

Verschiedene Schwermetallinhibitoren wie Pyrophosphat (3), Natriumazid, Thioharnstoff, Schwefelwasserstoff, hemmen den Sauerstoffverbrauch in geringem Grade (27). Doch betrifft die Wirkung nicht die eigentliche DO, sondern die 2. Oxydationsstufe. Wenn nämlich diese Stoffe zugesetzt werden, dann hört (bei Verwendung des Acetontrockenpulvers) der Sauerstoffverbrauch nach Aufnahme eines Atoms Sauerstoff auf und geht nicht wie üblich darüber hinaus. In ähnlicher Weise wirkt auch die Blausäure, doch ist deren Einwirkung so komplexer Art, dass diese in einem gesonderten Abschnitt behandelt wird.

Unter den Inhibitoren ragen an Bedeutung die organischen Basen, Vitamin B₁ und die Carbonylreagentien hervor. Sie werden im Folgenden einzeln angeführt.

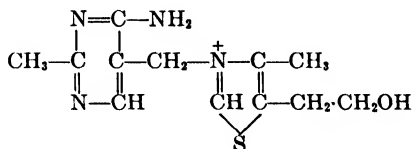
1. Organische Basen.

Wohl alle Abkömmlinge des Ammoniaks können bei genügend hoher Konzentration mit den Diaminen um die DO konkurrieren und dadurch diese hemmen. Dabei lassen Monoamine wie Methyl- und Amylamin nur eine geringe, Cholin (20) und Ephedrin (9) eine grössere und Guanidin (29) und Imidazol (23) eine sehr grosse Affinität erkennen. Bei den einfachen Guanidinen nimmt die Hemmung vom Guanidin zum Methyl- und asym. Dimethylguanidin (18) zu. Sehr gross ist auch die Hemmung durch die Diguanidine Arcain und Synthalin (18) (Tetra- bzw. Decamethylendiguanidin), die beide eine hypoglykämische Wirkung ausüben. Die Wirkung des Imidazol wird stark herabgesetzt, wenn an einer Seiten-Kette eine Carbonylgruppe sitzt (23).

Einige basische Farbstoffe wie Methylenblau (4), Pyocyanin und Toluylenblau (18) verhindern schon bei sehr viel kleineren Konzentrationen (10^{-4} -molar) als die übrigen Basen die Oxydation der Diamine. Möglicherweise hängt dieses Verhalten mit der Eigenschaft dieser Farbstoffe, Wasserstoff aufnehmen zu können, zusammen, da andere, als Redox-Indikatoren dienende Farbstoffe mit einem positiveren oder negativeren Normalpotential einen sehr viel kleineren Einfluss besitzen.

2. Vitamin B₁

Bei der DO gelang es zum ersten Mal, mit Aneurin (Thiamin) ein Ferment des Basenstoffwechsels zu beeinflussen. Aneurin ist ein Diamin, das als Abkömmling des Trimethylen-diamins betrachtet werden kann. Seine Affinität zur DO und damit seine hemmende Wirkung ist sehr gross. Seither wurden ähnliche Befunde für andere Basen abbauende Enzyme mitgeteilt (30, 31).



3. Carbonylreagentien

Alle bisher geprüften Carbonylreagentien hemmen die DO, zum Teil bei sehr kleinen Konzentrationen (10^{-5} -molar): Semicarbazid, Thiosemicarbazid, Hydroxylamin, Dimethylcyclohexandion (Dimedon), Natriumhydrogensulfit (6, 18), Kaliumcyanid (2, 27), Phenylhydrazin und die Girard'schen Ketonreagentien Hydrazidocarboxymethylpyridiniumchlor-

rid und Dimethylamidoessigsäurehydrazidchlormethylat (32). Die DO scheint somit eine für die Oxydation der Diamine notwendige Carboxylgruppe zu tragen.

Diese ausserordentliche Hemmbarkeit durch Carbonylreagentien kann zur Identifizierung der DO verwendet werden (22, 33). Sie trennt Sie vor allem von der Monoamin-oxydase, die auch durch die tausend- bis zehntausendfache Konzentration nicht beeinflusst wird (33a). Ähnlich wie die DO verhält sich die Histidincarboxylase gegenüber den Carbonylreagentien (32).

4. *Kaliumcyanid*

Schon bei der "Histaminase" wurde die grosse Empfindlichkeit gegen Cyanide festgestellt (2) und für die DO bestätigt (6); aber unter bestimmten Bedingungen wurde auch eine Aktivierung der DO gefunden (18). Die Einwirkung des Kaliumcyanids auf den enzymatischen Abbau der Diamine ist komplexer Natur und liess sich in einer eingehenden Analyse (27) in mindestens 5 Vorgänge auflösen, die teilweise früher in der Fermentchemie nicht bekannt waren:

(a) In der Fermentchemie ist Kaliumcyanid vor allem wegen seiner Eigenschaft, Schwermetalle zu entionisieren und dadurch Schwermetallfermente zu inaktivieren, bekannt. Es ist aber auch denkbar, dass Kaliumcyanid wegen seiner Fähigkeit, mit Carbonylgruppen Cyanhydrine zu bilden, enzymatische Prozesse beeinflussen könnte. Da die bisher untersuchten üblichen Schwermetallinhibitoren wirkungslos sind, dagegen alle und sehr verschieden gebaute Carbonylreagentien stärkste Hemmung verursachen, so kann mit Sicherheit angenommen werden, dass der Mechanismus der DO-Hemmung durch Kaliumcyanid auf einer Cyanhydrin- und nicht auf einer Komplexbildung beruht. Die Hemmung ist vollständig reversibel, wenn das Kaliumcyanid durch Zusatz von Brenzträubensäure oder durch Absorption in Lauge im Manometergefäss dem System wieder entzogen wird.

(b) Dieser eben erwähnte Entzug des Kaliumcyanids kann durch die DO-Reaktion selber erfolgen. Es entsteht, wie gezeigt wurde, beim enzymatischen Abbau der Diamine ein Aldehyd, der mit dem Kaliumcyanid reagiert. Wenn die Cyanidkonzentration so gewählt wird, dass eine geringe Oxydation möglich ist, dann nimmt diese autokatalytisch zu, weil immer grössere Mengen von Cyanid gebunden werden, bis dieses vollständig eliminiert ist und die Umsatzgeschwindigkeit normal geworden ist. Das ist genau dann der Fall, wenn pro Molekül Cyanid ein Atom

Sauerstoff verbraucht und demgemäss ein Molekül Substrat in den entsprechenden Aldehyd umgewandelt worden ist.

(c) Durch diese Cyanhydrinbildung mit dem entstehenden Aldehyd wird dieser aber aus dem Reaktionsgleichgewicht entfernt und die Reaktion beschleunigt, was bei geeigneten Cyanid- und Cadaverinkonzentrationen tatsächlich erkennbar wird. Diese Resultate bilden einen eindeutigen Hinweis auf die Reversibilität der DO und auf die Möglichkeit der Bildung von Diaminen durch dieses Ferment, auch wenn die Gleichgewichtslage unter den gewöhnlichen Bedingungen stark nach der Seite des Abbaus verschoben ist.

(d) Es wurde dargelegt, dass Stoffe wie Natriumsulfid und Thioharnstoff die 2. Oxydationsstufe der DO ausschalten. In gleicher Weise reagiert auch das Kaliumcyanid, aber im Gegensatz zur Reaktion I als Komplexbildner. Wenn Substrat- und Cyanidkonzentration so gewählt werden, dass durch den geschilderten Abfangprozess nicht alles Cyanid gebunden wird, dann hört die Reaktion nach Verbrauch eines Atoms Sauerstoff auf. Erhöhen wir aber die Substratkonzentration, sodass alles Cyanid eliminiert wird, so geht die Oxydation weiter. Indirekt hemmt damit Kaliumcyanid die eigentliche DO, weil in diesen Fällen das Reaktionsprodukt nicht auf dem normalen Weg durch Weiteroxydation aus dem Gleichgewicht entfernt wird.

(e) Wenn wir bei gleichbleibender Cyanidkonzentration die Cadaverinkonzentration steigern, dann nimmt paradoxerweise die Reaktionsgeschwindigkeit ab. Es handelt sich hier um den schon erwähnten Summationseffekt. Durch die Blockierung eines grossen Teiles der reagierenden Fermentgruppen tritt Eigenhemmung durch überoptimale Substratkonzentration in Erscheinung. Diese Reaktion ist nicht für Kaliumcyanid spezifisch (Kapitel VII).

5. Aktivatoren

Phosphat und Oxalat aktivieren, wahrscheinlich weil sie das hemmende Calcium entionisieren. Eine aktivierende Wirkung von Kaliumcyanid wurde im vorangehenden Abschnitt beschrieben.

Es wurde auch ein Aktivator von Enzymcharakter dargestellt (18): Wird eine Schweineniere mehrere Stunden mit Aceton behandelt, und der aus diesem Pulver gewonnene Extrakt gründlich dialysiert, so besitzt dieser nur eine sehr geringe DO-Aktivität. Aus Rattenleber lässt sich nach der für die Diaphorase (resp. Coenzym factor) gegebenen Vorschrift (34) ein Präparat gewinnen, das nicht dialysabel, hitzeempfindlich und

ohne die geringste DO-Aktivität ist. Setzen wir es zu den erwähnten Extrakt, so wird dessen Fähigkeit, Diamine zu oxydieren und zu desaminieren, um das Mehrfache gesteigert (Fig. 2.)

VII. Chemismus des enzymatischen Diamin-Abbaues

In Schweineniere- (25) und Känguruhleber-Extrakten (36) verläuft die Sauerstoffaufnahme mit gleichbleibender Geschwindigkeit, bis fast 2 Atome Sauerstoff verbraucht worden sind. Durch Fällung mit Ammonsulfat oder bei Behandlung der Organe mit Aceton hört dagegen die Sauerstoffaufnahme schon nach Verbrauch von 1 Atom Sauerstoff auf (Fig. 3) (22). Durch die angegebenen Prozeduren wird offenbar ein Ferment ausgeschaltet, das den Verbrauch eines 2. Sauerstoffatoms verursacht.

Da dieser Vorgang, der als 2. Oxydationsstufe bezeichnet wird, nicht die eigentliche DO betrifft, sei sie hier nur so weit behandelt, als sie für das Verständnis der DO nötig ist.

Die erste Oxydationsstufe entspricht einer mono-molekularen Reaktion (3, 22). Die Aufnahme eines Atomes Sauerstoff entspricht der Ablösung eines Molekels Ammoniak, und zwar auch bei Putrescin und Cadaverin, und die Inaktivierung der biologischen Wirkung des Histamins (22, 35). Das zweite Atom Sauerstoff dürfte, wenn es das unveränderte Histamin oxydierte, dieses nicht inaktivieren oder das unveränderte Cadaverin nicht desaminieren, sodass die Annahme, dass das Reaktionsprodukt der DO weiter oxydiert wird, viel wahrscheinlicher ist.

Von den in der Gleichung (Kapitel II) aufgeführten Reaktionsprodukten wurde der Aldehyd bis zu 30% als bisulfitbindende Substanz gefunden. Das Peroxyd wurde indirekt durch Sekundäroxidation von Aethanol nachgewiesen. Ausser Aethanol lassen sich eine grössere Zahl von Sub-

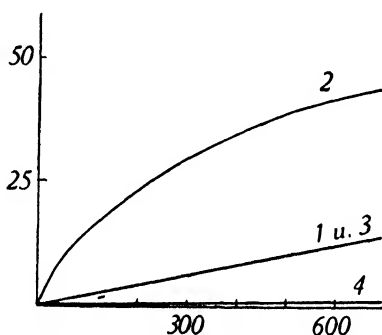


Fig. 2.—Abszisse: Minuten; Ordinate: mm.³ Sauerstoff; Ferment I Cadaverin 0.002-m., Aktivator 10 mg., Werte ohne Substrat subtrahiert. Kurve 1: Ferment + Cadaverin. Kurve 2: Ferment + Cadaverin + Aktivator. Kurve 3: Ferment + Cadaverin + 15 Min. auf 100° erhitzter Aktivator. Kurve 4: Aktivator + Cadaverin.

stanzen wie Brenztraubensäure, Harnsäure, Carotin (36) und das schon erwähnte Indigodisulfonat auf diese Weise oxydieren.

Dieser Aldehyd wird weiter oxydiert, offenbar zuerst zur entsprechenden Säure, da das pH der Fermentlösung auch bei guter Pufferung nach längerer Reaktionsdauer saurer wird. Beim enzymatischen Abbau des Histamins durch das an Fullererde adsorbierte Ferment wurde noch ein weiter oxydiertes Produkt mit einer Carbonylgruppe gefunden, das als kristallisiertes Dinitrophenylhydrazon abgeschieden wurde. Es enthielt 1 Atom Kohlenstoff weniger als das Histamin. Schliesslich entsteht beim Histamin ein schwarzer Farbstoff, dessen Bildung durch Sulfonamide wie Irgamid (37) verhindert wird.

Die Kurven, die die Abhängigkeit der Oxydationsgeschwindigkeit von der anfänglichen Substratkonzentration darlegen, zeigen beim Histamin

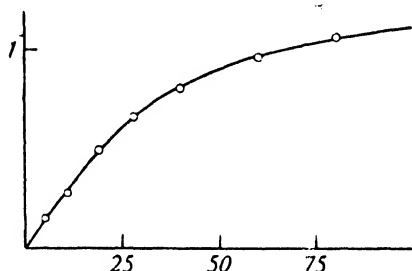


Fig. 3.—Oxydation des Putrescins an der Diamin-oxydase. Abszisse: Minuten; Ordinate: verbrauchte Sauerstoffatome pro Molekel Putrescin.

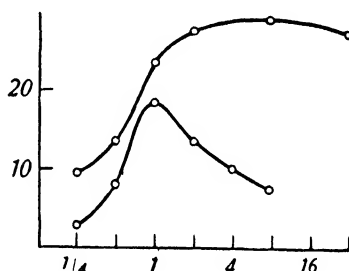


Fig. 4.—Aktivitäts- p_K -Kurve von Cadaverin- und Histamin-Diamin-oxydase. Obere Kurve: Cadaverin; untere Kurve: Histamin.

und Agmatin ein sehr scharf ausgeprägtes, beim Putrescin und Cadaverin dagegen ein kaum angedeutetes Optimum. Spermin und Spermidin nehmen eine Mittelstellung ein.

Die Ursache dieses wechselnden Verhaltens der aufgezählten Substratgruppen scheint so zu erklären sein, dass bei Histamin und Agmatin die Affinität der einen der beiden basischen Gruppen um das Mehrfache grösser ist als die der andern (Kapitel VI), beim Putrescin und Cadaverin aber ungefähr gleich. Wenn, wie es schon für andere Fermente angenommen wurde (38), zwischen der DO und ihrem Substrat eine doppelte Bindung stattfindet, dann muss es bei der Konkurrenz der beiden Gruppen des Histamins oder Agmatins um die entsprechenden Gruppen des Ferments zu einer bevorzugten Bindung der Imidazol- resp. Guanidgruppe und von

zwei Substratmolekeln an ein Fermentmolekel kommen (Fig. 3). Aus dem in Fig. 3 dargestellten Schema geht weiterhin hervor, dass ein Monoamin wohl an die DO gebunden und damit den Abbau eines Diamins verhindern kann (Kapitel VI), selber aber nicht oxydiert wird; denn nur ein Körper, der mit 2 basischen Gruppen an die beiden entsprechenden Gruppen des Ferments gebunden wird, bildet einen zerfallsfähigen Ferment-Substratkomplex.

Die Eigenhemmung durch überoptimale Substratkonzentration ist umso grösser, je grösser das Verhältnis der Konzentrationen der Substratmolekel zu der der freien Fermentgruppen ist. Dieses Verhältnis kann nicht nur durch Vergrößerung der Substratkonzentration, sondern auch durch Verkleinerung der Zahl der freien Gruppen des Ferments herbeigeführt werden. Das kann durch Blockierung dieser Gruppen durch Kali-

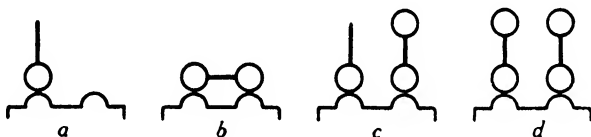


Fig. 5.—Schematische Darstellung der Bindungsverhältnisse zwischen Diaminen und der Diamin-oxydase. Die Kreise bedeuten die Amino- oder andern basischen Gruppen.

umcyanid (Kapitel VI), durch Cholin oder Semicarbazid (20) geschehen. Die hemmende Wirkung des betreffenden Stoffes wird dadurch vergrößert, dass ein Teil des Fermentes ausgeschaltet und die vorher optimale Substratkonzentration zu einer überoptimalen gemacht wird. Diese Erscheinung wird als Summationseffekt bezeichnet und lässt sogar bei Putrescin und Cadaverin die sonst kaum angedeutete Eigenhemmung durch überoptimale Substratkonzentration sehr scharf hervortreten (27). Es ist möglich und nicht unwahrscheinlich, dass dieser Effekt intra vitam der Regulation der Enzymaktivität der Organe dient.

VIII. Vorkommen der Diamin-oxydase

Zu der nachstehenden Tabelle (15), die die Arbeiten verschiedener Autoren zusammenfasst, ist ergänzend zu bemerken, dass beim Menschen in der Placenta (22, 39), Pancreas und Spermaplasma (40, 41) viel, in Prostata und Samenblasen weniger, in Spermatozoen, Hoden, Gehirn und Netzhaut (36) keine oder nur Spuren von DO sich

vorfinden. Das letztere gilt auch für Kuhmilch (36), für Herz, Magen, Haut, Blase und Urin des Hundes (2) und für Hefe (36).

Beim Säuger treffen wir die DO am häufigsten in Niere, Darmschleimhaut und Leber. Gewöhnlich ist in der Niere, vor allem in der Nierenrinde, wesentlich mehr als in der Leber vorhanden. Eine Ausnahme bildet das Känguruh. Die Nager weichen in mehrfacher Hinsicht von den übrigen Säugern ab. Bei den Vögeln ist umgekehrt meistens in der Leber die grössere DO-Aktivität als in der Niere vorhanden.

	Niere	Leber	Darmschleimhaut	Nebenniere	Blut	Lunge	Muskel	Milz
Mensch	++	++	+++	++	+	+		
Rind	+++	++	+++	—				
Schaf	+++	++						
Pferd	+++	++						
Schwein	++++	±				—		
Ratte	—	±	+++			+		—
Meerschweinchen	++	++	+		+	—		—
Kaninchen	±	±			—			
Hund	+++	±	+++	+	+	+	+	+
Katze	+++	+			+			
Jaguar	+++	?	++					
Känguruh	+	++++	++					
Huhn	+	+	+			—		—
Taube	++	++	+			+	+	++
Turmfalk (<i>Falco tinnunculus</i> L.)	++	++						
Ente	—	++						
Steinkauz (<i>Athene noctua</i> Scop.)	+	++						
Seidenhuhn (<i>Gallus bankiva</i> dom.)	?	+						
Elster	+	++						
Star	+	++						

Im Laufe der Ontogenese verändert sich bei Mensch, Rind und Star die DO-Aktivität verschiedener Organe (15). So ist in der Nierenrinde des neugeborenen Kindes keine DO nachweisbar, wohl aber im Nierenmark, während beim Erwachsenen der Gehalt in Rinde und Mark ungefähr übereinstimmt (9).

Eine starke Zunahme erfährt die DO des menschlichen Serums während der Schwangerschaft. Die Messung der DO des menschlichen Serums kann deshalb zur Schwang-

gerschaftsbestimmung verwendet werden. Sie wird entweder biologisch mit Histamin (33), oder rein chemisch mit Cadaverin durchgeführt (16, 17, 18, 19, 20, 21, 42). Diese Zunahme steht wahrscheinlich im Zusammenhang mit dem grossen DO-Gehalt der Placenta.

IX. Hormonale Einflüsse auf die Diamin-oxydase

Die Diamin-oxydase unterliegt der steuernden Wirkung von Hormonen. Das geht schon aus der Aktivitätsänderung während der Gravidität, die ihrerseits hormonal gelenkt wird, hervor. An erster Stelle ist die Nebennierenrinde zu nennen. Die Empfindlichkeit gegenüber Histamin wird bei Ratten durch Nebennierenextirpation stark gesteigert. Dieser schon längere Zeit bekannten Tatsache (43) entspricht die kürzlich gefundene Abnahme der Lungen-DO der Ratte (44). Da Histamin von parathyreoectomierten Hunden im Harn ausgeschieden wird, ist auch ein Einfluss der Nebenschilddrüse zu vermuten (45).

X. Biologische Bedeutung der Diamin-oxydase

Die DO steht mit ihren Substraten (Putrescin, Cadaverin, Agmatin, Spermin, Spermidin) und Hemmungsstoffen (Vitamin B₁, Guanidine) mit einer grossen Zahl von Stoffwechselvorgängen und Organfunktionen in Beziehung. Im Folgenden werden von diesen einige wenige herausgegriffen, bei denen anscheinend voneinander unabhängige Vorgänge durch die DO miteinander verknüpft werden.

(a) Im Säugerorganismus werden Histamin durch die Histidincarboxylase (49), durch Darmbakterien verschiedene Diamine und auf noch unbekanntem Wege die Polyamine Spermin und Spermidin gebildet. Normalerweise werden diese Stoffe so vollständig abgebaut, dass sie nicht im Harn erscheinen. Die DO ist also im Stande, diese Stoffe mit genügend grosser Geschwindigkeit zu oxydieren. Ausnahmen finden sich bei gewissen Schwangerschaftstoxicosen (46), bei parathyreoectomierten Hunden (45) und in manchen Fällen von Cystinurie, bei denen sich Putrescin und Cadaverin im Harn ausscheiden (47). Bei diesen Ausnahmen werden mehr Diamine gebildet als die DO bewältigen kann.

Das regelmässige Vorkommen der DO in der Darmschleimhaut muss wohl als eine Schutzeinrichtung des Organismus vor einer Überschwemmung mit bakteriell gebildeten Diaminen aufgefasst werden. Wirklich wurde in der Leber wohl Histamin, das der Körper selber synthetisieren kann, aber weder Putrescin noch Cadaverin gefunden (48). Die gleiche

Folgerung würde auch aus der Anwesenheit der Monoamin-oxydase in der Darmschleimhaut gezogen (47a). Eine ähnliche Funktion dürfte auch der DO der Placenta zum Schutze des Embryos zukommen.

Es wurde gezeigt (Kapitel VI), dass Vitamin B₁ möglicherweise eine Rolle im Diamin-Stoffwechsel spielt. So könnte die Wirkung der internen Aneurin- (Thiamin-) Therapie bei Neuritiden teilweise über die DO gehen. Aneurin blockiert mit seiner grossen Affinität die DO und verzögert den Histaminabbau. An Stelle des von aussen durch Jontophorese angewandten Histamins würde das vom Körper selber gebildete treten.

(b) Die DO ist in mehrfacherweise mit der Fortpflanzung verknüpft. Im menschlichen Sperma, in dem wie bei manchen Tieren auch ein DO-Substrat (Spermamin) auftritt, beeinflusst sie die Beweglichkeit der Spermien (41). Besonders auffallend ist der gewaltige Anstieg der DO zu Beginn der Schwangerschaft, der wohl mit der bekannten Schwangerschafts-Histidinurie, in Beziehung zu setzen ist, da Histidin die Muttersubstanz für die Histaminbildung ist. Die Intensivierung des Diaminstoffwechsels während der Gravidität wird auch durch das Vorhandensein der grossen DO-Mengen in der Placenta erkennbar. Wenn Diaminbildung und -abbau nicht aufeinander abgestimmt sind, sind die Folgen für den Körper verhängnisvoll, da für so wirksame Substanzen wie Histamin das Nichteinhalten der optimalen Konzentration zu verschiedenen Störungen führen muss. Gewisse Schwangerschaftstoxikosen wurden schon längere Zeit als Histaminintoxikationen angesehen (50). Die Befunde, dass im Harn von Eklampischen Histamin und im Serum eine abnorm hohe DO-Aktivität vorhanden sind (36), bilden eine starke Stütze für diese Vorstellung.

(c) Mit Pigmentierungsvorgängen scheint die DO in mehrfacher Hinsicht verbunden zu sein. So entsteht beim enzymatischen Abbau von Histamin ein melaninähnliches Pigment (Kapitel VII). Es liegt somit nahe, die bei der Schwangerschaft auftretenden Pigmentierungen auf die gesteigerte Tätigkeit der DO zurückzuführen. Bei Vitiligo, einer Pigmentierungsanomalie, ist die Serum-DO herabgesetzt (37). In vitro wird die Melaninbildung aus Dioxyphenylalanin gehemmt, wenn gleichzeitig ein Diamin (oder Monoamin) fermentativ abgebaut wird (37).

(d) Von Fermentreaktionen, die mit der DO verbunden sind, sei hier nur die Cholinesterase angeführt. Beide Fermente werden durch Vitamin B₁ gehemmt und das Produkt der Cholinesterase, Cholin, blockiert die DO, umgekehrt Histamin (51) Cadaverin und Agmatin (36) die Cholinesterase. Beide Fermente ändern während der Gravidität ihre Aktivität. Die DO nimmt im Serum der Frau zu, die Cholinesterase ab (52).

(e) Die DO ist vielfältig mit dem Zuckerstoffwechsel verflochten. Die Substrate Histamin, Agmatin, Spermin und die Inhibitoren Guanidin, Decamethylen-diguanidin (Synthalin) und Vitamin B₁ wirken auf den Blutzucker. Spermidin hemmt die Glukose-, Milchsäure- und Brenztraubensäure-dehydrierung (53), und Agmatin und vor allem Guanidin und Synthalin verhindern die Pasteur-Reaktion (54). Einige der aufgezählten Stoffe werden bekanntlich therapeutisch bei Diabetes verwendet.

Es müssen wohl tiefgreifende Änderungen im Zuckerstoffwechsel die Folge von Störungen des Diamin- und Polyaminstoffwechsels sein. Von diesem Gesichtspunkt aus gewinnen wir ein Verständnis für das häufige gemeinsame Vorkommen von allergischen Erkrankungen, die mit Histamin als ätiologischer Ursache in Zusammenhang gebracht werden, und Diabetes, vor allem mit dem sthenischen Überdruck-Diabetes (55), der pathogenetisch nicht auf einen primären Insulinmangel, sondern auf eine Überfunktion der Nebennierenrinde zurückgeführt wird. Es wurde erwähnt, dass dieses Organ die DO beeinflusst. Auch für das gemeinsame Auftreten von Cystinurie, die in schweren Fällen mit Diaminurie einhergeht, mit Diabetes (55), von Schwangerschaft und Störungen im Zuckerstoffwechsel ("Schwangerschaftsdiabetes"), oder Änderungen im allergischen Verhalten (56) bildet die DO möglicherweise das verbindende Glied.

XI. Therapeutische Verwendung der Diamin-oxydase

Ausgehend von der Annahme, dass bei vielen allergischen Krankheiten Histamin die auslösende Ursache sei, wurde versucht, die "Histaminase" therapeutisch auszunützen (57). Es wurde über erfolgreiche Anwendung bei Colitis ulcerosa, Kälteallergie, Rhinitis vasomotoria, Bronchitis fibrinosa, Serumkrankheit und andern Krankheiten berichtet. Teilweise wurden aber auch Misserfolge bei einzelnen dieser Affektionen, vor allem auch beim Asthma festgestellt (58). Auch die experimentelle Untersuchung über die Wirkung der DO-Präparate auf Vorgänge, die durch Histamin beeinflusst werden, oder bei denen Histamin frei wird, sind noch widerspruchsvoll. In der einen Versuchsanordnung verhinderte die mit der Duodenalsonde verabreichte DO die Magensaftsekretion, die nach einem Bad auftritt (59), bei einer andern konnten auch noch so grosse Mengen von DO, die parenteral zugeführt wurden, die Produktion von Magensaft nach einer Histamininjektion nicht hemmen (60).

Die perorale Applikation der DO bietet besonders grosse Schwierigkeiten für das Verständnis einer Wirkung auf den Histaminstoffwechsel.

Die Salzsäure des Magens, Pepsin und Trypsin zerstören in kürzester Zeit das Ferment (18, 61, 62). Auch wurde gerade in den Fällen, bei denen die DO-Therapie wirksam war, bei Colitis ulcerosa, keine vermehrte Histaminbildung im Darm, wohl aber bei Asthma gefunden (63).

Die Präparate, die bisher verwendet wurden, sind so komplexer Natur, dass es schwierig ist, zu beweisen, ob tatsächlich die DO oder nicht irgend ein anderer Bestandteil die beobachteten günstigen Effekte hervorgerufen hatten. Erst wenn experimentell sichergestellt sein wird, dass der DO-Gehalt und die Wirkung verschiedener Präparate parallel verlaufen und dass der DO-Gehalt unabhängig von der Herkunft des Fermentes von verschiedenen Tierarten oder Organen und unabhängig vom Grade der Reinigung allein entscheidend ist, darf ein entsprechender Kausalzusammenhang angenommen werden. Dass vor allem parenteral angewandte Fermente ausgiebig wirken können, dafür sprechen die Wirkungen der Schlangengifte, die zur Hauptsache fermentativer Natur sind, und des Fermentes, das in spezifischer Weise die Kapsel-Polysaccharide des Pneumococcus III spaltet und Mäuse gegen die millionfache tödliche Dosis zu schützen vermag (64).

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THE CHEMISTRY OF TEA-FERMENTATION

By

E. A. HOUGHTON ROBERTS

Cinnamara, P. O. Assam, British India

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I. Introduction

The discoloration of plant tissues following extensive mechanical damage is a frequently observed phenomenon, and one that has some considerable practical importance in addition to its intrinsic interest. The most familiar examples of the effect are the discolorations of fruits such as the apple and peach which follow bruising, and scientific inquiry has been largely directed toward the minimizing of such effects (1, 2). When tea-leaf is subjected to extensive mechanical damage the original bright green of the leaf is masked by a copper-red color developed as a result of the oxidation of tannins. This process, which is one of the chief stages in the manufacture of black tea, is generally referred to as tea-fermentation, incidentally a somewhat unfortunate term as there is no real analogy to anaerobic or bacterial fermentation.

In the fermentation of tea the main object is to ensure that as much as possible of the tissue is affected, and that no green unfermented leaf remains. The necessary damage is achieved by subjecting the leaf to a slight shearing force such as is obtained by rubbing between the palms of the hands. In the early days of tea manufacture the leaf was actually hand treated in this way but nowadays it is twisted in machines which are capable of handling about 300 lb. of leaf at a time. Before the leaf is thus mechanically rolled it is usually subjected to a preliminary withering process, which has the effect of reducing the moisture content of the leaf from about 77 to 65% under Assam conditions, although in some districts a much higher wither is employed. As a result of this wither the leaf can be twisted much more easily and unnecessary smashing up of the tissue, such as would attend the treatment of the turgid unwithered leaf, is avoided. The purpose of withering is therefore mainly one of physical preparation for the subsequent rolling, although some chemical changes occur which have their effect on the quality of the finished product.

Although fermentation is initiated with the first damage to the leaf it takes some considerable time to achieve the damage necessary to ensure complete fermentation. By the time the rolling process is completed, fermentation is already considerably advanced but to realize the necessary degree of oxidation of the tannins a further period of fermentation is necessary. The latter stages of fermentation are carried out by spreading the rolled leaf out on a flat surface where it is left until a satisfactory color has developed. The total time of fermentation, including the rolling period, usually varies from $2\frac{1}{2}$ to $4\frac{1}{2}$ hours. The fermented leaf is then rapidly dried in a blast of hot air at about 90°C .

It has long been recognized that the pigmentation of plant tissues following injury is due to the enzymic oxidation of polyphenols. The enzymic nature of tea-fermentation is indicated by the fact that a preliminary steaming of the leaf prevents the development of the fermentation reactions on subsequent injury. This inactivation of enzymes by heat is of importance in the manufacture of green teas where tannin oxidation must be avoided.

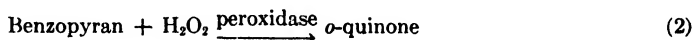
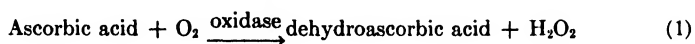
Once it was admitted that tea-fermentation was an enzymic process, it remained to be decided whether the enzymes responsible were those present in the leaf or whether the process was brought about by microorganisms. Various workers, notably Evans (3), have shown that bactericides, such as mercuric chloride, have no effect on the process. The best evidence against this latter view is that provided in these laboratories by Benton (unpublished observations) who has shown that there is no correlation between bacterial numbers and rate of fermentation. Bacterial development, except under conditions where it has been intentionally raised far above the extent normally encountered, has no significant effect upon the rate of fermentation. It may therefore be concluded that the process is catalyzed by oxidase systems already present in the leaf, and as will emerge from the following pages, this is confirmed by the successful oxidation of tea-tannins by leaf oxidase preparations.

II. The Properties and Nature of the Oxidizing Enzymes in the Tea-Leaf

The enzymic nature of tea-fermentation was recognized as early as 1893 by Bamber (4). In the early years of the twentieth century Aso (5), Nanninga (6) and Newton (7) observed oxidase activity in the tea-leaf while Mann (8) also showed peroxidase to be present. This latter finding was confirmed by Bernard and Welter (9). More recently Manskaya (10) has undertaken a more detailed study of the oxidizing enzymes in tea-leaf. After removal of the tannins by grinding the tissue with hide powder, high peroxidase activity was reported in the residue. It was stated that oxidase activity was either absent or very low but this claim was based on the absence of any direct oxidation of guaiacol. As this substance is not oxidized by oxidases in the absence of some quinone-producing substrate, such, for example, as tea-tannin, Manskaya's claims cannot be accepted.

The presence of an oxidase promoting direct oxidation of tannin in Ceylon tea-leaf was strongly indicated by Lamb's original findings (11, 12), and the work of Jones (13) in South India confirmed this. Our first results, however, seemed to confirm Manskaya, for Roberts and Sarma (14) showed that tea-leaf, ground with alcohol until free from tannin, contained no detectable oxidase but was very active as a source of peroxidase. This apparent absence of oxidase was also remarked upon by Deijs (15).

Later, work by the author (16) showed that the rate of tea-fermentation was in no way correlated with peroxidase activity. As the evidence then available seemed to show that peroxidase was intimately concerned in the oxidation of tannins during fermentation, it was suggested that the H_2O_2 necessary for peroxidase activity was produced as a result of enzymic oxidation of some respiratory carrier and that the enzyme responsible for this oxidation was inactivated by alcohol. Reasons were advanced (17) for identifying this enzyme with ascorbic acid oxidase, and it was shown that on this hypothesis the reactions involved in tannin oxidation bore a strong resemblance to those suggested by Huszák (18) for the oxidation of substrates in peroxidase plants, in which dehydroascorbic acid and *o*-quinones behaved as H-acceptors in dehydrogenations.



Tea-tannin as a catechin derivative would provide the necessary benzo-pyran.

The above scheme requires that the oxidation of ascorbic acid precede that of tea-tannin, whereas it has been shown quite conclusively that ascorbic acid oxidation by fermenting tea-leaf is effected by oxidized tea-tannin (19). There is reason to believe that the tea-oxidase can also oxidize ascorbic acid directly (20) but its rate of oxidation is certainly much lower than that of tea-tannin. Lamb and Sreerangachar (21) have also shown that ascorbic acid cannot function as a carrier in the oxidation of polyphenols by the tea-oxidase. The same authors query the direct oxidation of ascorbic acid and ascribe it to traces of polyphenols adsorbed by the active enzyme.

As the various tea-tannins contain either the catechol or the pyrogallol grouping it is tempting to identify the oxidase with polyphenol oxidase but its inactivation by alcohol or acetone and its low solubility in water conclusively negatives this idea. The possible identity of the enzyme with cytochrome oxidase was first suggested by Lamb and Roberts (22) and further evidence in favor of this view is contained in publications from this laboratory (19, 20). The fundamental fact upon which this hypothesis is based is the discovery by Sreerangachar (23) that the oxidase is associated largely with water-insoluble particles. The inactivation of the enzyme by organic solvents, its enhanced activity in oxygen and the wide range of substrates oxidized, all speak for its identification with cytochrome oxidase, although Lamb and Sreerangachar (21) cannot confirm our findings that substrates other than *o*-dihydroxyphenols are oxidized by the tea-enzyme.

If we are to accept the view that cytochrome oxidase is the enzyme responsible for tea-fermentation it follows that the tannin is oxidized by oxidized cytochrome and not directly by oxygen. There is some evidence available which suggests that a carrier of this nature is concerned in the oxidation of tannins.

Mixed substrate tests (24) indicate that with mixtures of tea-tannin and catechol, oxidation of the former substrate is probably nearly complete before catechol oxidation begins. It is considered that this behavior is to be expected if a carrier is involved, but not if it were a question of competition between two substrates for the active enzyme surface.

It has been shown (25) that when suspensions of finely ground tea-leaf in water are shaken, the uptake of oxygen is affected by the dilution of the tissue in rather a novel manner. This rate, per unit quantity of tissue, increases with the dilution by quite a considerable amount. Diffusion factors and effects due to substrate concentration cannot account for this and I am forced to the conclusion that at high dilutions a greater propor-

tion of a carrier is in the reduced state, and that the rate of oxidation is in consequence higher.

Recently Lamb and Sreerangachar (21) have strongly criticized the cytochrome hypothesis. In my opinion, however, these workers expect too great a correspondence between cytochrome systems in plant and animal tissues. The tea-oxidase certainly differs from the cytochrome oxidase of animal tissues in several important ways (24).

(a) Although the greater part of the oxidase is insoluble in water, aqueous extracts free from cellular matter can be obtained, particularly from withered leaf (21), and this suggests a greater ease of peptization of the vegetable oxidase.

(b) The enzyme is not completely destroyed by alcohol or acetone at 0° C., about one-third of the activity being retained.

(c) Oxidation of hydroquinone and *p*-phenylenediamine is less marked than that of polyphenols.

(d) *M*/100 cyanide is required for maximum inhibition.

(e) The *pH* optimum is at 5.4, and enzymic oxidation of tannin is low at *pH* 3.5 and 7.2.

The above properties are not peculiar to the tea-oxidase. Thus Okunuki (27) showed that the oxidase of lily pollen, while oxidizing cytochrome-*c*, did not oxidize hydroquinone. Little is known about the *pH* optima of plant oxidases but Gräff (28) has shown that the optimum *pH* range for the Nadi reaction is on the acid side of neutrality for leaf tissue but on the alkaline side for animal tissues. My own results (24) show that the oxidation of tannins in a mince of leaf from *Eugenia balsamea* is optimum at *pH* 5.5. Again there are comparatively few data available for the cyanide sensitivity of plant oxidase systems. The position was reviewed by Deb and Roberts (20) and further data have since been published (24) which show that in some half dozen cases *M*/100 cyanide, or an even greater concentration, is required to suppress oxidase activity.

The cytochrome system was first demonstrated in the tissues of higher plants by Keilin (29) and again later by Yakushiji (30). Recent work by Bhagvat (31) and Hill and Bhagvat (32) has confirmed these observations. Bhagvat examined 18 species and spectroscopic evidence of the presence of cytochrome was found in all of them. In no case was cytochrome absent and in some tissues the intensity of the cytochrome spectrum is such as to justify the belief that the greater part of the respiration is associated with the cytochrome oxidase. Among the plant sources rich in cytochrome were maize embryos and my own results show that the respiratory system in maize embryos is incompletely inhibited by *M*/100 HCN (24). This may be taken as a strong indication that a plant cytochrome system may have a low sensitivity to cyanide, as is required by my contention that cytochrome oxidase is responsible for tea-fermentation.

More convincing evidence is afforded by the results of CO poisoning on the respiration of plant tissues. Kempner (33) has shown that the inhibition by CO of the respiratory activity of several plant tissues is reversed by light, and that tobacco leaf was among the tissues so affected. The reversal by light of CO inhibition has also been established for the young leaf of the carrot by March and Goddard (34) and for an enzyme preparation from tea-leaf by Deb and Roberts (20). This behavior is characteristic of hematin systems, and the only oxidase known to show this effect is the cytochrome oxidase. Incidentally, the relative affinities of the respiratory enzymes of tea and carrot tissue for oxygen and CO is similar to that found by Warburg for yeast. All three of the above systems have been shown to be relatively slightly inhibited by cyanide. Our own results (20, 24) show that $M/100$ HCN is necessary for complete inhibition of both tobacco respiration and tea-oxidase systems, and Marsh and Goddard (34) have shown that carrot leaf respiration is only 70% inhibited by $M/1000$ HCN. These results, therefore, show that plant oxidase systems may have very close resemblances to the animal cytochrome system and yet have very much lower sensitivity toward cyanide.

Unfortunately we have only circumstantial evidence for both the role of cytochrome in tea-fermentation, and for the belief that plant cytochrome systems have characteristics of their own which distinguish them from those of animal tissues. Confirmation requires methods for the study of the cytochrome system in the leaf which at the moment have not been developed.

The chlorophyll in the leaf prevents a direct spectrometric approach and the inactivating effect of organic solvents on cytochrome oxidase rules out methods in which the tissue is first treated with alcohol or acetone. Mechanical separation of the enzyme from chlorophyll by centrifuging the ground-up tissue at high speeds is unlikely to be attended with much success as it appears that the oxidase is closely associated with chloroplastic material (26).

The alternative method of approach is to study the effect of additions of cytochrome-c to the plant oxidase system. Lamb and Sreerangachar (21), having failed to accelerate tannin oxidation in this way, conclude that the tea-oxidase is probably not a cytochrome system, but although I can confirm this observation I do not agree with the deduction. It is possible that the system may already contain sufficient of the carrier to enable the enzyme to catalyze tannin oxidation at its maximum rate. There are also reasons for believing that tea-tannin may have an inhibitory effect upon cytochrome preparations from foreign tissues, while the tea-cyto-

chrome may be resistant to such an effect. It was recently shown by Roberts and Sarma (35) that the oxidases of the two species, *Camellia thea* (Link), *i. e.*, tea, and *Eugenia balsamea*, were specific for their own tannins. The oxidase of one had comparatively little activity toward the tannin of the other. If tea-tannin has an inhibitory effect upon an oxidase from another plant species it is not unlikely that it will have a similar inactivating effect upon ox-heart cytochrome, which must be considered a foreign protein.

Finally there are two further observations which confirm the belief that the cytochrome system is active in the tea-leaf. Although it has so far been impossible to establish the presence of cytochrome oxidase in the tea-leaf, experiments by Miss Bhagvat (31) have shown it to be present in the basal portion of the stem. This makes it at least probable that the leaf also contains a cytochrome system.

It has been shown by Tuzimura, Ako and Sasaki (36) that riboflavin is present in Japanese green teas. As a flavin is a necessary intermediary in the transfer of oxygen from cytochrome to many dehydrogenase systems its presence in the tea-leaf is to be expected if cytochrome oxidase is responsible for tea-fermentation and respiration. It may be recalled that von Euler and Dahl (37) found flavin in the germinating seeds of several species since shown by Bhagvat (31) to contain the cytochrome system.

Considering all the above evidence it is my opinion that there is a very strong case for the identification of the tea-oxidase with cytochrome oxidase. There do appear to be distinct differences in properties between plant cytochrome systems and those of animal tissues but the evidence put forward by Lamb and Sreerangachar (21) is not sufficiently clear cut to cause me to abandon this hypothesis.

One consequence of the identification of the tea-oxidase with cytochrome oxidase is to query the role played by peroxidase. There is no reason to believe that hydrogen peroxide is produced in the oxidation of cytochrome in which case some other source is required. The experiments of Lamb and Sreerangachar (21) appear to show that peroxidase does not participate in tea-fermentation. Thus although tannin oxidation by tea-enzymes is enhanced by the addition of hydrogen peroxide, 10^{-4} M HCN, which has but little effect on tea-fermentation, reduces the rate of tannin oxidation to the same level as that in the absence of peroxide. The conclusion seems unavoidable that under normal circumstances peroxidase plays no role. However, these experiments were carried out in relatively dilute solution and it may be that peroxide concentration would then be too low to be utilized by peroxidase. It was shown in this laboratory (25) that catalase

had no inhibitory effect upon fermentation when the bruised tissue was suspended in excess water, while earlier work (14) had shown a considerable inhibition when the tissue was only moistened with water. However, the Ceylon workers have observed a case where leaf from one particular bush cannot undergo fermentation unless mixed with oxidase from a sample of normally fermenting leaf. This leaf contains the normal amount and is rich in peroxidase. It is clear from this that peroxidase alone cannot be held responsible for tea-fermentation, and it is doubtful whether it has any role at all.

Although peroxidase is widely distributed in plant tissues there appears to be no definite evidence of its playing any important part in plant metabolism. In the past it has been rather glibly assumed that it utilizes peroxide in promoting oxidations but there appears to be little real reason for believing that this is its true function. The possible identification of peroxidase with dihydroxymaleic-oxidase by Swedin and Theorell (38) may throw some light on this difficult question.

III. Chemical Changes Accompanying Tea-Fermentation

As a result of extensive analyses over many years it is possible to account for the great majority of the components of the tea-leaf, as the following table shows. These figures, for Assam leaf, are expressed as percentages of the total dry weight.

Tannins	22.0
Other polyphenols	0.2
Protein N \times 6.25	17.2
Caffeine	4.3
Other N \times 6.0	7.2
Reducing sugars (glucose)	3.5
Starch	0.5
Pectins	6.5
Crude fiber	27.0
Ether soluble matter	2.0
Ash	5.6
<hr/>	
Total	96.0

Organic acids, malic and oxalic, account in part for the deficiency.

These figures are for fresh leaf. In the course of withering there is a substantial decrease in carbohydrates. Starch disappears but reducing sugars may be but little affected. Protein breakdown takes place and there is a corresponding increase in "other" N, which is probably largely to be identified with amino acids.

In fermentation of withered leaf the main changes are those concerning the tannins. There is further oxidation of glucose but total N is unaffected although there may be some amide production.

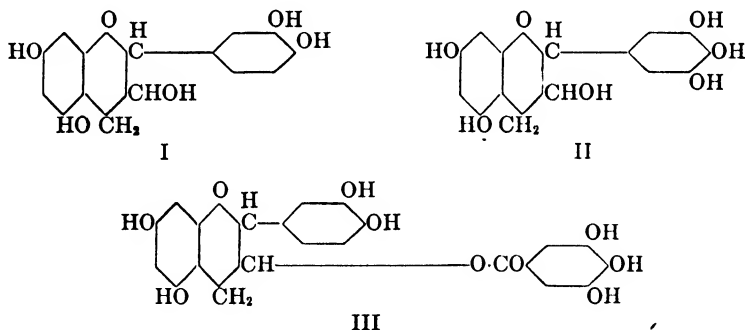
Changes in the pectins are probable but these have been but little investigated and, finally, there is a considerable reduction in ether-soluble-matter.

In addition to these bulk changes, fermentation affects the interrelations of the components of the leaf, particularly the combinations of the tannins with the nitrogenous compounds. This results in considerable changes in the solubility in water and alcohol of the leaf components.

The above chemical changes will now be considered in more detail.

1. The Tannins

The extent of our present knowledge of the constitution of tea-tannins has been summarized in two recent communications by Harrison and Roberts (39) and Deijs (40). In Assam the tannins of green leaf appear to consist mainly of the simpler condensation products of epi-catechin (I) and gallo-catechin (II) whereas in Java the galloyl ester of epi-catechin (III) is probably the most important.



From the chemical structure of the tea-tannins it is evident that an *o*-quinone is likely to be the first product of oxidation and this is confirmed by its properties as a H-acceptor in the oxidation of ascorbic acid and carbohydrates. Further evidence for *o*-quinone production is furnished by the preparation by Lamb and Sreerangachar (21) of an aniloquinone when tea-tannin is oxidized enzymically in the presence of aniline.

If fermentation is allowed to proceed to completion it is found that the oxygen consumption is equivalent to an uptake of one atom of oxygen per molecule of tannin, together with the amount consumed in converting the

glucose metabolized into CO_2 (39, 24). Despite the unstable nature of *o*-quinones as a class there is no evidence of any oxidation of the tannins beyond this stage. It therefore follows that the tannins, after oxidation to the *o*-quinone, must undergo some further change and it is believed that this change is a condensation-polymerization of the type visualized by Freudenberg (41), in which the secondary carbinol grouping of one molecule condenses with the phloro-glucinol nucleus of another. As the reaction product still possesses the two groupings necessary for further condensations of this type, this process is theoretically capable of almost limitless repetition. The catechins themselves show little tendency to undergo such condensations except at elevated temperature or in slightly alkaline or strongly acid solutions, but, according to Freudenberg, if the catechin is first oxidized (presumably to the *o*-quinone) condensation follows oxidation with great rapidity.

The more or less highly condensed tea-tannin oxidation products are much stronger tannins than the tannin of green tea-leaf and are capable of precipitating gelatin from aqueous solution, and of combining with the protein of the leaf itself. The tannins in an aqueous extract of fermented tea differ therefore in the following respects from those in a similar extract of green leaf.

- (1) The catechol grouping will be oxidized to an *o*-quinone.
- (2) The tannins will be more highly condensed.
- (3) Some of the tannins by reason of their combination with leaf protein will no longer be water-soluble.

On account of (1) and (3), volumetric methods of estimating tea-tannin will show a reduction in the tannin titer as a result of fermentation. It has also been shown by Barua and Roberts (42) that the extent of condensation may affect the tannin titer. The methods hitherto in use, *viz.*, the well-known Lowenthal method and the alkaline iodine method of Shaw (43), both have the disadvantage that oxidation, by permanganate or iodine, is to some indeterminate end-product. Thus although epicatechin requires 62 equivalents of oxygen for complete oxidation to CO_2 , in the Lowenthal and Shaw methods 7.1 and 16.7 equivalents, respectively, are consumed in the oxidation of one molecule of tea-tannin. As a result of condensation it would be expected that the tannins would become more resistant to oxidation. In both these empirical methods slower oxidation means less oxidation and we may expect that the fall in the tannin titer will be greater than can be accounted for by oxidation and combination with protein.

Barua and Roberts (42) have demonstrated that these expectations are realized and have further shown that with a method based on complete oxidation of the tannins by alkaline permanganate to CO_2 , the fall in the tannin titer is determined by extent of oxidation and combination with protein only. This new method thus allows us for the first time to determine the amount of tannin in fermented teas with some precision. The Lowenthal method, because of the uncertainty introduced by condensation, cannot give a true idea of the amount of tannin in black teas, despite its official recognition by the Association of Official Agricultural Chemists.

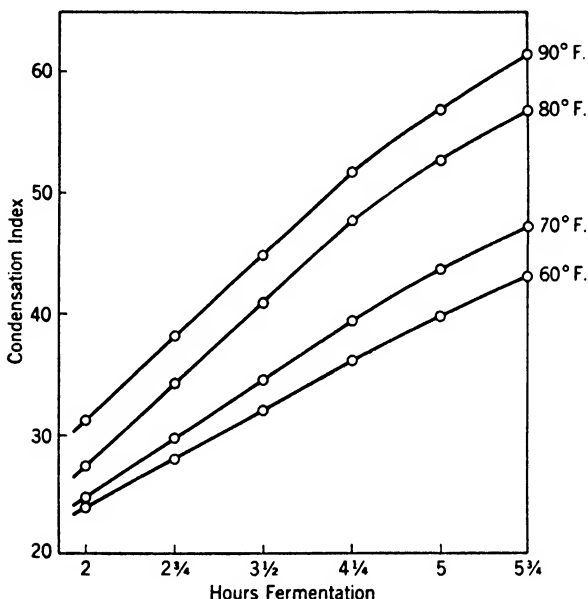


Fig. 1.

The investigation of the condensation of tannins during tea-fermentation is complicated by our lack of knowledge of polymerization kinetics in general. However, it has been possible to devise an empirical method of measuring the degree of condensation. The water-soluble tannins in fermented tea may be separated into three fractions by successive precipitation with 1% H_2SO_4 and saturation with salt. It has been shown by Harrison and Roberts (39) that the H_2SO_4 precipitate may be considered to be the most highly condensed fraction, and its estimation by the method of Barua and Roberts (42) gives us a measure of the extent of condensation. The titer in ml. 0.1 M KMnO_4 of this fraction per g. of dry fermented tea is referred to as the condensation index (unpublished observations).

Unlike the extent of oxidation of tannins, which is optimal at about 28° C. (21, 26), and which is not materially increased by extending the time of fermentation beyond 3½ to 4 hours, the condensation index increases regularly with the duration and the temperature of the fermentation process (Fig. 1).

The changes in the condensation indicated by the figures above allow us to account for many of the chemical changes observed that accompany the fermentation process. The full data, which include eight repeats of four temperatures and six times of fermentation, cannot be quoted here and will be reported elsewhere. An outline of the results obtained is given in the following table. The analytical figures are expressed as percentages of the dry weight of the tea.

	Green leaf	Tea fermented 2 hrs. at 60° F.	Tea fermented 5¼ hrs. at 90° F.
Water-soluble solids	45.6	43.2	38.5
Water-soluble tannin	22.0	19.2	15.1
Alcohol-soluble solids	47.0	38.0	25.5
Partition coefficient (ethyl- acetate water)	Large	0.90	0.35
Condensation index	Nil	23.8	62.0

The decreases in water-soluble solids, water-soluble tannins and alcohol-soluble solids are all highly significantly correlated with the condensation index, as is the difference between alcohol- and water-soluble solids. The decrease in the extractability of the tannins by ethyl acetate as measured by the partition coefficient is also significantly correlated with the condensation index. Further there is a close correlation between water soluble solids and water-soluble tannins.

These results may be compared with those of Castagnol and Doan-ba-Phuong (44), who carried out a series of analyses of Indo-China teas at various stages of manufacture and found that both the extracts in cold water and hot alcohol decreased very considerably as the length of the fermentation period increased. The residue from these two extracts was then taken up in boiling water and the amount thus extracted was found to increase with increasing extent of fermentation.

My interpretation of the above results is as follows:

With increasing condensation of the tannins, combination with leaf-protein is favored, so that both soluble tannins and total soluble solids are reduced. The reduction in the latter will largely be determined by that of the former.

In addition to combining with protein, to form complexes which can only be broken down by hydrolysis with alkali (39), the condensed tannins can also combine with the other nitrogenous substances of the leaf,

particularly caffeine. These complexes are completely broken down at the temperature of boiling water but resist boiling acetone or alcohol, and cold water. The fall in the alcohol-soluble solids and cold water extractives, therefore, is due to combination of tannins with protein and caffeine and extraction of the residue with boiling water is successful because of the break-up of the tannin-caffeine complex. The steady increase in water-soluble minus alcohol-soluble solids with fermentation may also be put down to this caffeine complex and the decreased extractability of tannins by ethyl acetate from the cooled aqueous infusion is a measure of the strength of the attachment of caffeine to the higher condensed tannins.

2. *Carbohydrates*

During the fermentation of tea CO_2 continues to be evolved, although the R. Q. is much lower than in green leaf. The CO_2 produced is almost exactly equivalent to the loss in reducing sugars, expressed as glucose (24) so that there seems little doubt as to its origin.

3. *Nitrogenous Compounds*

The carbohydrate content of tea-leaf appears to be sufficiently high to make it unnecessary for any demands to be made on the protein matter for respiratory material. Nevertheless a slight oxidation of amino acid with production of ammonia is indicated by the results of Castagnol and Doan-ba-Phuong (44) and this is confirmed by my own observation that amido-N is increased slightly as a result of fermentation.

Castagnol and Doan-ba-Phuong quote an extensive series of results for nitrogen fractions at different stages of fermentation but unfortunately their results cannot be accepted. Amino-N is determined according to Sørensen, which method is very inaccurate in the presence of tea-tannin, the error involved increasing as fermentation progresses. The estimation of amino-N in tea has never been satisfactorily accomplished as all methods so far tried are subject to very large errors in the presence of tea-tannin.

4. *Ether-Soluble Matter*

With Assam leaf the ether-soluble matter is reduced to about half its original value as a result of fermentation. This decrease is in part due to chlorophyll degradation but the magnitude of the change is such that true fats must also be affected in the fermentation.

Castagnol and Doan-ba-Phuong (44) have established similar changes in Indo-China tea but here the original ether-soluble matter was much higher than in our leaf. These workers fractionated the ether-soluble matter into petroleum-ether and water-soluble portions and made chromatographic studies of the various fractions during the course of fermentation. There remains no doubt from these observations that although the ether-soluble matter is very heterogeneous, all the constituents of this fraction undergo quite extensive changes as a result of fermentation.

These changes may be of importance in the development of flavor in tea during fermentation. The constitution of the substances present in the essential oil of tea is such as to indicate their origin from lipid, rather than carbohydrate or protein sources, and it is possible that they arise through a secondary oxidation of the fats by the *o*-quinones formed in the oxidation of the tannins.

IV. The Mechanism of Tea-Fermentation and Its Relation to Respiration

Although early workers on tea-fermentation made little attempt to explain the mechanism of the process, discoloration in other plant tissues received some attention. Thus Szent-Györgyi and Vietorisz (45) suggested that the oxidation of polyphenols at the surface of a freshly cut potato tuber resulted in the production of *o*-quinones whose function was to protect the tissue from bacterial infection at the site of the damage. It has been observed by Benton in this laboratory that extensive mechanical damage to the tea-leaf results in a sharp drop in the number of bacteria on the leaf and it is my belief that this drop is due to the bactericidal properties of the *o*-quinone of tea-tannin. After fermentation for an hour or so the greater part of the *o*-quinone molecules will have condensed and the bactericidal properties will have been lost. In conformity with this Benton finds that bacterial numbers may rise again toward the end of the fermentation.

Another view on the mechanism of these pigmentations was that of Sutter (46) who believed that oxygen diffused faster into the tissues as a result of the mechanical damage, and that, in consequence, *o*-quinones were produced at a rate faster than they could be reduced by dehydrogenase systems.

It has been noted by many that the oxidases concerned in polyphenol oxidation are also the oxidases primarily concerned in respiration. The relation of the two processes, however, remained obscure. In 1934 a team of Russian biochemists working with Oparin (47) made an attempt to in-

interpret tea-fermentation as "disorganized" respiration. Their conclusions which are not always consistent, are summarized below.

According to Manskaya (10) oxidizing and reducing processes are in equilibrium in the intact cell, but mechanical damage leads to an inactivation of the reducing processes. The respiratory pigments are therefore no longer reduced but undergo further oxidation to form stable brown pigments which can no longer serve as H-acceptors. As a result of the preponderance of oxidizing processes organic peroxides were assumed to accumulate and these peroxides were believed to be utilized by peroxidase in furthering the oxidation of tea-tannin. Kurssanov (48) considered the accumulation of oxygen to take place in the early stages of fermentation and the oxidation of tannins to be a secondary process which made use of this accumulated oxygen. It was shown in accordance with the above hypothesis that tea-leaf after two hours' rolling continued to deepen in color when transferred to a CO₂ atmosphere. This observation, however, is equally well explained by my condensation theory, for after two hours' mechanical rolling a considerable proportion of the tannins are oxidized, and condensation would be expected to continue, with consequent darkening in color, in the complete absence of oxygen.

Kurssanov interpreted the approximate 50% fall in the Lowenthal tannin titer, which takes place as a result of fermentation, as a fermentative breakdown of the tannin molecule, and then postulated oxidation of fission products such as catechol and gallic acid. To account for the gradual slowing down of the process he assumed that the enzymes are inactivated by the tea-tannin. As he believed that tea-tannin was capable of completely destroying the enzymes (49) it was suggested that water-soluble proteins, developed in the withering, protected the enzymes, but that as a result of the gradual precipitation of these proteins during fermentation this protective effect eventually disappeared.

These hypotheses of Kurssanov do not stand experimental tests. Throughout fermentation there is no detectable breakdown in tannins to simpler products. The slowing down of the process is due to the completion of tannin oxidation, as the addition of fresh substrate stimulates oxygen uptake to nearly its original level (17). Further, at temperatures below 25° C., enzymic inactivation at the end of the process is observed to be slight (26). Finally Kurssanov's figures for water-soluble protein extractable at 40° C. are open to grave doubt. No water-soluble protein can be detected in tea-leaf extracts, and the variations in water-soluble nitrogen on which Kurssanov bases his hypothesis are almost entirely due to the formation of tannin-caffeine complexes not extractable by water at 40° C.

Despite the imperfections of this work the results are of value. A

definite step forward was made in connecting fermentation with respiration. In particular it was suggested that mechanical damage so upset the balance of oxidations and reductions that irreversible oxidation of tannins became the main reaction in the system. This suggestion has been fully confirmed by our own work, which will now be dealt with in some detail.

In investigating the mechanism of the fermentation process manometric methods have been extensively used. The fresh or withered tea-leaf is ground to a fine paste and portions of this are then suspended in distilled water. Buffer need not be added as the buffering power of the tissue is sufficient. On account of the relatively short times during which uptakes are measured (1–2 hours), and the bactericidal nature of the tannin oxidation products, inaccuracies due to bacterial development are hardly to be expected. In confirmation of this it is found that the presence of large numbers of bacteria have no significant effect upon the rate and extent of tannin oxidation as determined manometrically.

It is found that the rate of uptake and the R. Q. are affected by the dilution of this suspension (25) but there is nothing to suggest that this involves any fundamental difference in the mechanism.

No matter how fine the state of division of the tissue there is always some output of CO_2 during the fermentation. The possibility that the CO_2 is produced by intact cells which are respiring normally may be rejected, for if we assume that the CO_2 is formed only in undamaged cells the oxygen consumed for complete oxidation of the tannins in the damaged cells amounts to 1.44 atoms O per molecule, a most unlikely result (24). In addition the rate of CO_2 formation falls within one hour to very low levels despite ample reserves of carbohydrate in the tissue, while in the intact leaf CO_2 production persists for several days with but little falling off in rate (20).

All our observations indicate that CO_2 production, which arises from carbohydrate oxidation, is secondary to tannin oxidation. Considering different commercial varieties of tea it has been shown that although the QO_2 values may differ considerably, the R. Q., for suspensions of 200 mg. tissue in 3 ml. of water, remains constant at 0.37 (17) as shown in Fig. 2. It will also be observed that CO_2 production runs strictly parallel with O_2 uptake and that once the oxidation of tannins approaches completion the rate of CO_2 formation falls to a very low level. This figure may also serve to show the general nature of the O_2 uptake and CO_2 output curves of fermenting tea-leaf, when suspended in water.

From these results it appears probable that in the finely ground tea-leaf oxidation of carbohydrates depends upon the formation of the *o*-quinone

of tea-tannin, and that a proportion of this oxidation product is able to function as a H-acceptor in dehydrogenations of carbohydrates or their breakdown products. The R. Q. in this case will be a measure of the proportion of *o*-quinone molecules which undergo reduction instead of condensation-polymerization to the brown pigments characteristic of fermented tea.

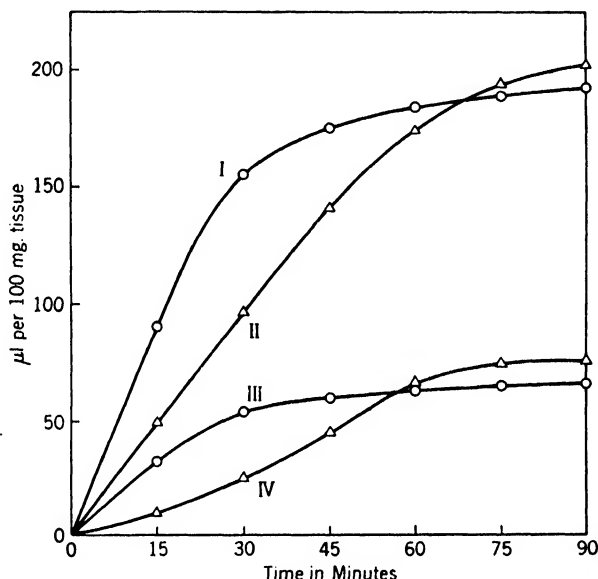


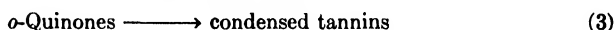
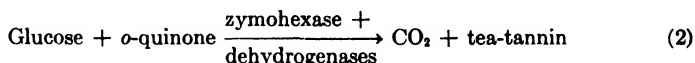
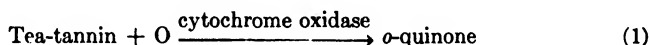
Fig. 2.

- I. Fast fermenting variety. O_2 uptake.
- III. Fast fermenting variety. CO_2 output.
- II. Slow fermenting variety. O_2 uptake.
- IV. Slow fermenting variety. CO_2 output.

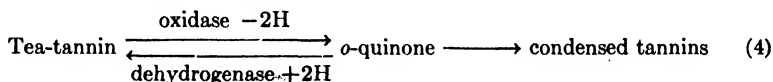
Addition of ascorbic acid to fermenting tea inhibits both carbohydrate oxidation and tannin condensation until sufficient oxygen has been consumed to effect complete oxidation of the ascorbic acid (17). The brown color characteristic of fermented tea begins to develop only after the whole of the ascorbic acid has been oxidized. This provides clear evidence of the necessity for intermediate formation of *o*-quinone both for carbohydrate oxidation and for the condensation of tannins. It is also found that in non-tanniferous leaves, such as *Tropaeolum*, fine grinding of the tissue

leads to almost complete suppression of respiratory activity and very little CO_2 production is observed (35).

The evidence for the role of *o*-quinones in effecting the oxidation of carbohydrates in finely ground leaf tissue admits of little doubt and we may write down the main chemical changes in tea-fermentation as follows:



The reactions may be summarized in the one equation



and the process will continue until the whole of the tea-tannin has been converted into condensation products.

The assumption that partial inactivation of the dehydrogenases by mechanical damage is responsible for the disorganization of respiratory activity, and hence for the irreversible oxidation of tannins, is insufficient to explain the facts. There must be some dehydrogenase activity to account for the secondary oxidation of carbohydrates in tea-fermentation, yet in non-tanniferous leaves an equivalent amount of damage leads to almost complete suppression of carbohydrate oxidation.

It has been shown by Deb and Roberts (20) that in tea-leaf sufficiently damaged so as to undergo complete fermentation, anaerobic fermentation cannot be observed. Partial inactivation of dehydrogenases cannot account for this so we must assume that in tea-fermentation a carrier, or carriers, linking the oxidase and dehydrogenase systems must be inactivated. As phenomena allied to tea-fermentation are also observed in tissues containing polyphenol oxidase, which does not require a flavin to link it to dehydrogenases, the carriers inactivated by the mechanical damage must be codehydrase I and II.

The effect of the damage to the tissue is such that both normal respiration and anaerobic fermentation are more or less completely suppressed, but in the presence of *o*-quinones some carbohydrate oxidation may take place. As, according to Dixon and Zervas (50), *o*-quinones require a coenzyme to link them to a dehydrogenase, we have to account for the apparent activity of coenzymes in finely ground leaf tissue when polyphenols are present.

To account for this apparent discrepancy it is necessary to consider the extent of mechanical damage required to result in complete oxidation of the tannins. After the manufacture of tea, microscopic examination reveals that comparatively few of the plant cells are destroyed, yet oxidation of the tannins up to 95% completion may be realized. It is important to realize that the tea-leaf is not so much crushed or pounded as subjected to shearing forces during the rolling period. Phillis and Mason (51) have shown that slight shearing forces on the cotton leaf have a marked effect on the composition of the continuous phase of the cytoplasm and on its permeability to solutes, and it is my opinion that disorganization of the cytoplasm, rather than rupture of the cell wall, is all that is required to effect the changes we recognize in tea-fermentation (24, 52).

We may consider the equilibrium of oxidation-reduction processes within the plant cell to depend upon the organization of proteins and phosphatides at an interface. Adsorption of substrate and coenzyme at specific points on a mosaic of protein and phosphatide is probably necessary for normal respiratory processes, and distortion of this membrane by shearing forces will decrease very considerably the number of active centers suitable for such adsorption. On this hypothesis the result of the mechanical damage will be to reduce the effective concentration of the coenzymes, and both respiration and anaerobic fermentation will be correspondingly affected.

Another effect of the shearing forces on the cytoplasm is to increase its permeability, and tannins are now no longer confined to the vacuole but may penetrate into the cytoplasm. Oxidation of the tannins to *o*-quinones follows and any coenzymes dispersed through the tissue will be oxidized in turn. The oxidized coenzyme may now function as a H-acceptor without having first to be adsorbed at a specific point on the protein-phosphatide surface, and will be able to effect a certain amount of oxidation of carbohydrates. The concentration of oxidized coenzyme will depend upon that of the *o*-quinone, and therefore we find (25) that as the dilution of the tissue suspension is increased, *o*-quinone concentration is decreased, and the R. Q. falls, owing to the slower rate of carbohydrate oxidation. The curve connecting the rate of CO₂ production with the dilution of the tissue suspension is very similar to that found for the variation of dehydrogenase activity with coenzyme concentration.

Although the above picture of tea-fermentation is admittedly speculative, it does account for all the experimental facts that have been accumulated in the last few years. It is assumed that carbohydrate oxidation in the tea-leaf follows an essentially similar path to that found in other tissues, and that coenzymes are concerned in the transfer of hydrogen from res-

piratory substrates to oxygen. The assumption is also made that the organization of metabolic processes within the cell is determined by the relative positions of protein and phosphatide molecules in relatively fragile membranes. It is admitted that there is no proof that either of these assumptions holds for the tea-leaf, but it may be argued that similar mechanisms to these have been established, or made very probable, for so many biological tissues that their application to the tea-leaf is justified.

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HETEROTROPHIC ASSIMILATION OF CARBON DIOXIDE*

By

C. H. WERKMAN AND H. G. WOOD

Ames, Iowa

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I. Introduction

In 1935 heterotrophic assimilation of carbon dioxide was advanced by Wood and Werkman (1) as a definite and experimentally supported concept. They stated, "It has been established with several species of *Propionibacterium* that the total carbon dioxide liberated during fermentation of glycerol plus that remaining in the form of carbonate is less than the original carbon dioxide added as carbonate. This decrease is believed to result from utilization of carbon dioxide by the bacteria during their dissimilation of glycerol. Carbon and oxidation-reduction balances support this view." The unexpected finding of carbon dioxide utilization by such typically heterotrophic organisms as the propionic acid bacteria had been

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first obtained some two years previous, but the unexpected nature of the results led to additional experiments in order to obtain convincing and, if possible, conclusive proof. It was for this reason that the authors in their initial proposal of heterotrophic utilization of carbon dioxide took a definite stand and have remained firm in their pronouncement notwithstanding considerable doubt and criticism expressed in private communications and in print.

The concept of heterotrophic utilization of carbon dioxide was first proposed at the Spring (1935) Meeting of the North Central Branch of the Society of American Bacteriologists in connection with studies on the fermentation of glycerol by bacteria belonging to the genus *Propionibacterium*. These bacteria do not form sufficient carbon dioxide from the glycerol to mask the uptake of carbon dioxide. Therefore, in a medium containing carbonate to neutralize the acids formed from glycerol, i. e., propionic and succinic with a trace of acetic, determination of the carbon balance indicated that the carbon dioxide at the end of the experiment was not equivalent to that of the original medium in the form of carbonate, and that the products of fermentation contained more carbon than was present in the glycerol fermented.

Table I taken from the original work of Wood and Werkman (2) clearly shows that carbon dioxide was utilized by four species of the heterotrophic propionic bacteria used in the experiment. It was pointed out at this time (1936) that:

"The fact that chemical analysis shows a decrease of carbon dioxide (accountable as carbonate carbon dioxide) is, perhaps, proof enough of carbon dioxide utilization. However, the carbon and oxidation-reduction balances furnish additional evidence."

The authors then continued (1936), "This observation (carbon dioxide utilization) requires a reinterpretation of previous results. Investigators have not considered the possibility of carbon dioxide utilization in constructing schemes of dissimilation. If one considers the limited number of bacteria which have been shown to utilize carbon dioxide and also that such forms (autotrophic) differ markedly from the propionic acid bacteria, failure to consider the possibility of carbon dioxide utilization may be understood." The principle of heterotrophic carbon dioxide utilization was again presented before the Second International Congress of Microbiology, meeting in London during the Summer of 1936. It was not, however, readily accepted and opposing comments were made.

It is significant that the same authors (4) made the following comment in 1938 regarding the utilization of carbon dioxide by animal tissue.

"Krebs and Johnson (1937) have recently shown that citric acid is synthesized by avian tissue from oxalacetic acid and some unknown compound. It is possible that this synthesis involves utilization of carbon dioxide."

TABLE I
DISSIMILATION OF GLYCEROL BY PROPIONIC ACID BACTERIA

Culture	Glycerol fermented per liter, mM.	CO ₂ utilized per 100 mM. of fermented glycerol, mM.	Products per 100 mM. of fermented glycerol			Carbon recovery		Oxidation-reduction index	
			Propionic acid, mM.	Acetic acid, mM.	Succinic acid, ‡ mM.	Basis-glycerol plus CO ₂ , %	Basis-glycerol only, %	Basis-glycerol plus CO ₂	Basis-glycerol only
49W	212.6	37.7	55.8	2.9	42.1	101.2	114.0	1.081	2.550
34W	209.0	43.2	59.3	2.0	34.5	93.1	106.6	0.925	2.270
52W*	112.0	20.0	78.4	5.9	8.7	94.6	101.0	0.918	1.386
11W†	218.4	1.1	89.3	2.6	3.9	96.5	96.8	1.135	1.162
15W	176.4	12.3	78.4	5.8	7.8	89.1	92.6	1.047	1.376

* 7.0 mM. of lactic acid produced per 100 mM. of fermented glycerol.

† 0.5 mM. of lactic acid produced per 100 mM. of fermented glycerol.

‡ Succinic acid identified by melting point and mixed melting point.

The experimental proof of carbon dioxide assimilation by animal tissue came in 1940 through the work of Evans and Slotin (3).

Since the isotopes of carbon have become available for use as tracers of fixed carbon dioxide, there has been a tendency to disregard the work done previously. It is true that with the advent of the tracer technique, detection of the fixation of carbon dioxide and its behavior in metabolism have been facilitated; nevertheless, fixation by heterotrophic forms already had been clearly demonstrated by quantitative data obtained with the propionic acid bacteria. Moreover, since all the products were aliphatic carbon compounds of two or more carbon atoms, fixation in a carbon to carbon linkage was shown to occur. Location of the fixed carbon among the products and its position within the molecule was a matter of speculation at that time. The isotopic investigations have been of particular service in clearing up these latter points.

Wood and Werkman (4) showed an equimolar relationship between the carbon dioxide fixed and the succinic acid formed, and found that inhibition of fixation by sodium fluoride (5) resulted in a corresponding reduction in succinic acid. As a result the proposal was made that the succinic acid was the result of a C₂ and C₁ synthesis. Pyruvic acid was suggested as the possible C₃ compound since it could be isolated from the fermentation (6).

This was essentially the situation at the beginning of 1940 when isotopes of carbon first became available.

Autotrophism and Heterotrophism

It is desirable at this point, and before detailed consideration of the phenomenon, to define in the light of present knowledge the expression "heterotrophic carbon dioxide assimilation." The photosynthetic utilization of carbon dioxide has been known for nearly a hundred years but less generally known to occur is the process referred to as chemosynthesis in which carbon dioxide is utilized by an organism employing "chemical energy" in contradistinction to "radiant energy," to reduce the carbon dioxide to form a product of assimilation. Previous to the discovery of the utilization of carbon dioxide by heterotrophic forms, chemosynthesis referred to the utilization of carbon dioxide by a group of organisms known as chemo-autotrophs, discovered in 1890 by the eminent bacteriologist, Sergius Winogradsky (7). Winogradsky established the existence of chemo-autotrophic bacteria which grow and reproduce in a wholly inorganic medium in the dark, *i. e.*, they contain no photosynthetic pigment. The energy required to build cell substance and to carry on metabolism is obtained from relatively simple chemical reactions involving the oxidation of such inorganic compounds as ammonia and nitrite in the case of the nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*, respectively) or sulfur compounds in the case of the non-photosynthetic sulfur bacteria. Hydrogen sulfide is oxidized to free sulfur by *Beggiatoa*, and sulfur to sulfate by *Thiobacillus thio-oxidans*. Hydrogen gas is oxidized by *Carboxydomonas oligocarbo-philis*, methane by *Methanomonas methanica* and certain organisms such as *Didymohelix* and *Crenothrix* may oxidize Fe^{++} or Mn^{+} to Fe^{+++} or Mn^{++} . In all cases carbon dioxide is, of course, reduced, generally along with oxygen of the air. The autotrophs are, in large measure, aerobic forms or reduce nitrate. Their nitrogen is generally obtained from ammonium salts or other inorganic salts such as nitrates or nitrites, but the important point is that carbon requirements of these bacteria are satisfied wholly by carbon dioxide.

The metabolism of the autotrophs is either (a) relatively simple or, more likely, (b) the organisms are able to synthesize the essential complex substances of the nature of vitamins (coenzymes) which must be supplied to the heterotrophs.

The existence of chemo-autotrophic forms of life is frequently not appreciated, to wit, the following statement,

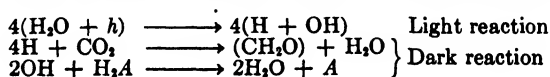
"It is generally known to scientists that photosynthesis is the synthesis of organic matter in green plants with the help of sunlight, and that *this process is the only source of organic matter existing on earth*" (8).

In similar relationship stands heterotrophic carbon dioxide fixation. Thus the idea has become fixed in mind that the assimilation of carbon dioxide is a process uniquely limited to photosynthesis by green plants.

Autotrophs may be photosynthetic, rather than chemosynthetic, such as certain of the sulfur bacteria (*Thiobacteriales*) which contain chlorophyll. Bacterial photosynthesis was confirmed by van Niel (9) after considerable controversy initiated by the original work of Engelmann (10) in 1883. These investigations constitute probably the first evidence for the existence of a photosynthetic process among bacteria. Engelmann found that the red pigmented (purple sulfur) bacteria possess a well-defined absorption spectrum and congregate in portions of the spectrum identical with those absorbed. Engelmann concluded that the pigment plays an essential role in the metabolism of these bacteria.

Winogradsky's original conception of autotrophism envisaged only the chemosynthetic aspects. He could not explain satisfactorily the role of hydrogen sulfide and light required for the growth of the photosynthetic purple sulfur bacteria (*Thiorhodaceae*). It was difficult to explain the light energy requirement in view of the oxidation of hydrogen sulfide to sulfur or sulfate, inasmuch as no rational reason was at hand for the two apparently independent sources of energy. Molisch's (11) discovery of the *Athiorhodaceae*, organisms which require organic substances as hydrogen donators to replace hydrogen sulfide or water in the case of the *Thiorhodaceae* or typical green plants, respectively, further confused the problem of bacterial photosynthesis until the investigations of van Niel offered a rational explanation and cleared the way for a better understanding of bacterial photosynthesis. Kluyver and Donker (12) in 1926 had suggested that with the purple sulfur bacteria hydrogen sulfide functions as a hydrogen donator replacing water in the typical green plant photosynthesis.

Van Niel (13) represents bacterial photosynthesis by the following equations:



H_2A is usually hydrogen sulfide although molecular hydrogen, organic H_2 -donators, such as fatty acids, or sulfur oxides may serve, depending on the species of purple bacterium used. In all photosynthesis water is the original source of hydrogen which is ultimately responsible for the reduction of carbon dioxide. In the case of the typical green plants the two "OH" groups form a peroxide which is decomposed into oxygen and

water. With bacteria the "OH" is reduced, for example, by hydrogen sulfide by the purple sulfur bacteria to form sulfur and water. For further details the reader is referred to the review by van Niel (13).

The general concept of photosynthesis as portrayed by van Niel is important in that the explanation of the dark reaction which results in the reduction of carbon dioxide may involve the same type of reduction as described by Wood and Werkman (4) for the heterotrophic assimilation of carbon dioxide and represented by: $\text{CO}_2 + \text{CH}_3\text{CO}\cdot\text{COOH} \longrightarrow \text{COOH}\cdot\text{CH}_2\text{CO}\cdot\text{COOH}$. This point will be further discussed. The function of light is to form active hydrogen; from here on photosynthesis may bear a close analogy to heterotrophic assimilation of carbon dioxide as proposed by Wood and Werkman.

In contrast to the autotrophic bacteria are the heterotrophic organisms which require a source of carbon more complex than carbon dioxide, *i. e.*, they are unable to utilize carbon dioxide (and now must be added) as a sole source of carbon. In view of the work of Wood and Werkman (1, 2, 14), Slade, *et al.* (15), Carson and Ruben (16), Ruben and Kamen (17), Barker, *et al.* (18), and others, the distinction between autotrophs and heterotrophs is becoming less evident; however, the terms are useful and carry a practical meaning. It is not possible at present to express in clear terms an explanation of the implied difference between an autotroph and a heterotroph; it remains a question just why heterotrophic forms require complex carbon sources when they are able to utilize carbon dioxide. It may be that inability of heterotrophic bacteria to use carbon dioxide as a sole source of carbon is linked with an inability to synthesize a certain molecular structure essential in their metabolism. On the other hand, this suggestion may be questioned, inasmuch as no such specific structure seems required by heterotrophs.

The differentiation of heterotrophs and autotrophs on the basis of carbon assimilation is difficult to apply in practice. The first difficulty is to determine whether the carbon (*e. g.*, from carbon dioxide) is assimilated. What constitutes assimilation? It is generally defined as the incorporation or conversion of nutrient material into body substance. If, then, the carbon of carbon dioxide is found at one stage in the protoplasm of the organism, assimilation clearly has taken place; if the carbon is found in a molecule of an enzyme active in metabolism, this would be accepted, probably by all, as assimilation. On the other hand, if the carbon is found in an excretion product, a question may be raised. Possibly a thermodynamic approach is to be preferred, but here again certain difficulties may arise in differentiating exergonic from endergonic reactions.

From the standpoint of mechanism, the difference between a typical heterotroph and a typical autotroph, as judged from present studies, is that the heterotroph can bring about a carbon to carbon linkage if one component of the linkage is organic, but it cannot repeat the process to form a linkage in which both components originate from inorganic carbon. In the case of the autotroph this can be done, however, and adjacent carbons can be inorganic. This only tells what the difference is but does not answer the fundamental question of why there is this difference. There are, however, certain borderline cases. For example, it is not to be concluded that certain organisms will not be found which require the presence in the medium of a substance of the nature of a vitamin which is essential for an autotrophic type of synthesis. An organism, *Clostridium acetium*, isolated and described by Wieringa (19), is able to live an essentially chemo-autotrophic existence when an unknown organic constituent of Dutch mud, which certainly cannot furnish appreciable energy, is added to the inorganic medium. Since the organism apparently reduces carbon dioxide by molecular hydrogen to form acetic acid, sufficient energy is available and only a coenzyme-like substance is required, and this is provided by the mud. In this case we have the picture of a typically autotrophic organism losing the property of synthesizing an essential organic constituent which must be supplied in the medium. The loss of the property in this case is a first step toward heterotrophism. The organism can, in fact, use sugars and function as a heterotroph.

It is likely that various organisms manifest heterotrophism for different reasons. One may be heterotrophic because it is unable to oxidize an inorganic substrate to provide the energy required to assimilate carbon dioxide, whereas inability to synthesize essential growth factors may force heterotrophism on another. It is not important and quite impossible to draw a sharp line of demarcation. An example of an essentially heterotrophic form requiring organic compounds as a source of carbon, and yet possessing the synthetic properties of an autotroph, is the organism recently isolated by Barker (18), *Clostridium acidurici*, which attacks purine compounds such as uric acid, xanthine and hypoxanthine anaerobically to form cell substance, ammonia, carbon dioxide and acetic acid. By the use of radioactive carbon dioxide (18) it was shown that the acetic acid was synthesized from carbon dioxide and that the fixed carbon occurred in both the methyl and carboxyl groups. While it was not possible to prove that individual molecules contained the fixed carbon in both groups since in any one molecule fixation may have occurred in only one group, nevertheless it seems quite likely that fixation does occur in both groups in indi-

vidual molecules. This organism may be an example of a heterotroph forming a carbon chain from C_1 compounds, *i. e.*, an autotrophic property.

Both *Clostridium acidurici* and *Clostridium aceticum* are examples of intermediate forms and manifest their autotrophism particularly by their ability to synthesize a carbon to carbon linkage from the C_1 compound, carbon dioxide. The heterotrophic utilization of carbon dioxide has not been proved, as yet, to be an essential step in cellular metabolism and may be no more than a vestige of autotrophism incapable of providing the carbon requirements of heterotrophic bacteria. Later discussion, however, will point out the probable importance of the utilization in the metabolism of the cell.

There is no evidence to indicate that chemo-autotrophic bacteria do not utilize carbon dioxide by the same or similar mechanism employed by the heterotrophs. Inasmuch as carbon dioxide is the sole source of carbon for the autotroph, it is to be expected that all the carbon to carbon linkages formed will comprise atoms originating from carbon dioxide, whereas probably only a small fraction of the typically heterotrophic assimilation employs carbon from carbon dioxide. Urgently needed are studies on autotrophic bacteria employing tracer carbon dioxide, preferably $C^{18}O_2$. The use of isotopes has opened new methods of attack and the whole problem of intermediary metabolism needs intensive study.

Probably the earliest suggestion that carbon dioxide plays an active role in heterotrophic metabolism was the result of the work of Novak (20) and Smith (21). The necessity for carbon dioxide became apparent in the case of *Brucella abortus* which was found to grow more readily, particularly if freshly isolated, when grown in the presence of an aerobe (*Bacillus subtilis*); although the effect was first attributed to decrease in oxygen tension, it was soon found to be due to an increased tension of carbon dioxide. Rockwell and Highberger (22) from a study of bacteria, yeasts and fungi, ventured the suggestion that heterotrophic microorganisms utilize carbon dioxide in their metabolism. Winslow, *et al.* (23), and Gladstone, *et al.* (24), examined the subject more closely. Carbon dioxide-free air bubbled continuously through liquid cultures of a large number of aerobes, and anaerobes prevented or greatly retarded growth, whereas when ordinary air was used growth was normal. The early work was largely qualitative and the effect of the carbon dioxide was frequently ascribed to its physical behavior. Thus until 1935 our ideas regarding the role of carbon dioxide in cellular metabolism were largely limited to the photosynthetic and chemosynthetic processes of the autotrophs.

It was mentioned that with the discovery of heterotrophic carbon dioxide assimilation, differentiation between the autotrophs and the heterotrophs became less distinct. It is not, however, implied that formerly a sharp line of demarcation was drawn between the groups. It was early recognized that intermediate forms occurred, and they were called facultative hetero-

trophs, if they were able to grow in an inorganic medium but tolerated the presence of organic constituents, especially in small concentration, and facultative autotrophs if organic material was preferred but growth could take place in its absence in a mineral medium. Even the early distinction between the autotroph and the heterotroph was appreciated to be a matter of convenience and rested in considerable degree on the assumption that heterotrophs were unable to assimilate carbon dioxide.

Inasmuch as the differentiation of autotrophs and heterotrophs is based on nutritional requirements of the organisms, it is convenient to visualize a spectrum (Fig. 1) in which the autotrophs and heterotrophs represent the

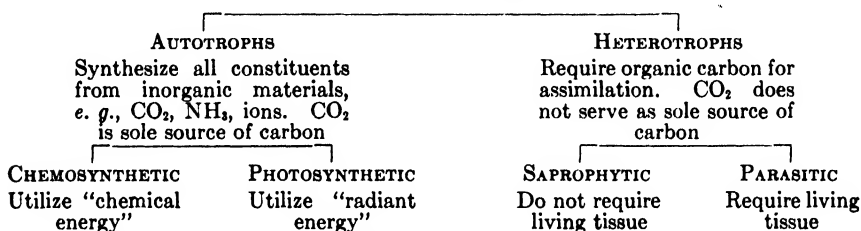


Fig. 1. Classification of organisms based on nutritional requirements.

two extremes and between these occur an indeterminate number of intermediate forms. The *Athiorhodaceae*, photosynthetic bacteria discovered by Molisch (11), apparently are unique in that they require organic compounds but in this case the organic substance serves simply as a hydrogen donor in a manner not clear, for the photosynthetic reduction of carbon dioxide. The carbon chain is not dissimilated and the fractions then assimilated to form cellular material. These bacteria are essentially autotrophic; however, in the present system of classification, their requirement of organic compounds would place them nearer the heterotrophs. A finer distinction might be made on the basis of function performed by the organic compound required. Further examples of intermediate forms are the organisms discovered by Wieringa (19) and by Barker, *et al.* (18).

There are, however, discernible differences in the type of function performed by carbon dioxide in cellular physiology. It may serve (1) simply as a hydrogen acceptor, or (2) it may be incorporated to form more complex carbon compounds in the cell, *i. e.*, assimilated by the creation of a carbon to carbon linkage. The two types have not been differentiated heretofore. The studies of Woods (25), Barker (26) and Hes (27) illustrate the first type, and although a utilization of carbon dioxide by heterotrophic organisms is involved, the same implications do not result as in investigations in

which heterotrophic assimilation has been shown to occur, *i. e.*, creation of a carbon to carbon linkage. This point will be discussed in further detail.

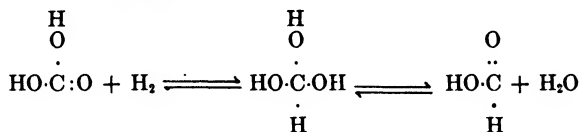
II. Mechanism of Heterotrophic Carbon Dioxide Fixation by Bacteria

No attempt will be made to give detailed consideration to all articles that have appeared. Only those that have a direct bearing on the mechanism of fixation will be discussed. The recent reviews (28, 29, 30) should be consulted for additional information. It is clear that the present consideration of the mechanism of heterotrophic fixation of carbon dioxide by bacteria will not offer a final solution to the problem raised by the discovery. Investigation of the mechanism has only recently begun in earnest, and it is true that future observations may change the general picture. Nevertheless, an outline of the present position should be of value in realizing shortcomings of present ideas, and in pointing out the next steps to be taken. It is hoped that the present review will be of value in investigations on animal physiology, a field in which the results of bacterial fixation of carbon dioxide have become only recently so pertinent. It is likely that extensive application of the fundamental facts discovered in bacterial physiology will be made to animal physiology. In fact, some applications have already been made, and these will be discussed. Thus far, most of the work on the actual mechanism of fixation of carbon dioxide has been done with bacteria, and particularly with the propionic acid bacteria. For this reason studies on the linkage of carbon atoms with carbon dioxide have been largely confined to the C_3 and C_1 addition. There are fixations in a number of other heterotrophic fermentations which fundamentally may be examples of C_3 and C_1 addition but the evidence is not clear at present. The mechanisms of bacterial fixation of carbon dioxide, therefore, will be considered under the following heads: (1) fixation of carbon dioxide not involving carbon to carbon linkage; (2) fixation of carbon dioxide involving carbon to carbon linkage. Under the latter will be discussed (a) C_3 and C_1 addition and (b) miscellaneous fixation reactions.

A. FIXATION OF CARBON DIOXIDE NOT INVOLVING CARBON TO CARBON LINKAGE

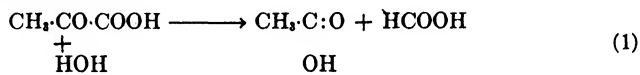
Formation of Formic Acid.—The reduction of carbon dioxide to formic acid by gaseous hydrogen with *Escherichia coli* has been clearly shown (25) and it presumably occurs with other bacteria that contain hydrogenylase,

i. e., produce carbon dioxide and hydrogen from formic acid. The exact kinetics of the reaction are not known, perhaps:

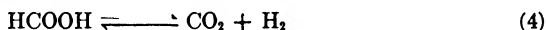
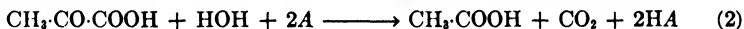


The enzymes have not been isolated or purified so as to permit a detailed study of possible coenzymes or carriers that may be involved. The reaction may be more complicated than pictured and, perhaps, include phosphorylation.

A problem of some importance is the mechanism of formation of formic acid, particularly whether it is usually formed by reduction of carbon dioxide. It is generally accepted that formic acid originates from pyruvic acid by a hydroclastic split:



and that carbon dioxide and hydrogen are formed from the formic acid. It seems just as likely that the reaction may occur stepwise as follows:



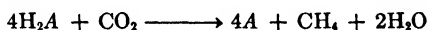
A in the above reactions functions as a hydrogen carrier. Accordingly formic acid would not be a direct intermediary product of pyruvic acid breakdown but would result by reduction of carbon dioxide. The hydroclastic reaction thus would be only a special case of pyruvic acid breakdown in which carbon dioxide was the hydrogen acceptor. The donator function of the pyruvic acid would be the same as in the dissimilation of pyruvic acid by a number of organisms to form acetic acid and carbon dioxide, *e. g.*, the aerobic dissimilation studied by Lipmann (31) and Barron (32), the dismutations of Nelson and Werkman (33) and Krebs (34), and the formation of acetic acid from pyruvate in the propionic acid fermentation (35, 36). The hydroclastic reaction was originally proposed to account for formation of formic acid from pyruvate before it was known that carbon dioxide is an active metabolite. Therefore, consideration has not been given to the reduction of carbon dioxide. Reactions (2) and (3) appear to account for the facts as readily as does reaction (1). For example, *Eberthella typhi* would be able to reduce carbon dioxide to formic acid

with the proper hydrogen donator but not with gaseous hydrogen. In all fermentations in which formic acid is formed in the presence of labelled carbon dioxide, the acid should contain isotopic carbon, even though reaction (4) did not occur. This point should be investigated further.

Formation of Methane.—Foster and his co-workers (28) have described the methane fermentation as

“One of the most interesting and up to now best understood of the biological processes utilizing carbon dioxide. . .”

Whether the process is considered well understood depends largely on the point of view. If one is comparing the old concept of the fermentation with the new concept, then our present understanding appears favorable. According to the present concept (26), methane fermentations of all types may be considered as a process of oxidation in which carbon dioxide acts as a hydrogen acceptor:



H_2A is the oxidizable molecule and A the oxidation product. H_2A may be ethyl alcohol and be oxidized to acetic acid, *e. g.*, *Methanobacterium omelianskii*, or it may be acetic acid and be oxidized to carbon dioxide as by *Methanosarcina methanica*. The reaction has been proved in the case of *Methanobacterium omelianskii* but with *Methanosarcina methanica* the proof of the reduction of carbon dioxide to methane as a result of oxidation of acetic acid is yet considered inconclusive (37) although quite likely.

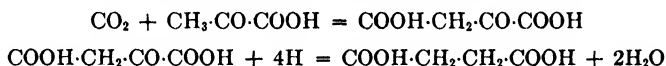
On the other hand, when one views the methane fermentation from the standpoint of understanding the actual mechanism of the reduction of carbon dioxide to methane, it must be admitted that we are almost completely in the dark. The intermediate steps are completely unknown, and a set of reactions for a possible explanation of the process has not been ventured. According to Barker (38) formic acid is not an intermediary in the process since it would not replace carbon dioxide in the fermentation by *Methanobacterium omelianskii*. It is evident that much important work is yet to be done before we have any understanding of the reactions which take place in the conversion of the most highly oxidized form of carbon to its state of greatest reduction.

B. FIXATION OF CARBON DIOXIDE INVOLVING CARBON TO CARBON LINKAGE

1. C_3 and C_1 Addition

Evidence That Succinate Is Formed by Fixation of Carbon Dioxide.—The first evidence of fixation of carbon dioxide by C_3 and C_1 addition was

obtained by fermentation of glycerol with the propionic acid bacteria (2, 4). It was established that for each mole of succinic acid formed from the glycerol one of carbon dioxide was fixed, *i. e.*, there was a net decrease in carbon dioxide of the system and in amounts equivalent to the succinic acid formed. When the fermentation was conducted in phosphate buffer in the absence of carbon dioxide little or no succinic acid was formed. It was, therefore, clear that carbon dioxide played an important role in succinic acid formation. Since the succinic acid (C_4) was formed from the glycerol (C_3) in amounts equivalent to the consumed carbon dioxide (C_1), the synthesis by C_3 and C_1 addition seemed the most probable mechanism. Pyruvic acid was proposed as the probable intermediary C_3 compound of the fixation reaction because it could be easily isolated from the fermentation liquor by the addition of sulfite to fix carbonyl compounds (6).



It was recognized that the equivalence of succinic acid formed and the carbon dioxide utilized might be the result of the stoichiometric demands of the oxidation-reduction balance, succinic being oxidized and carbon dioxide reduced. But this explanation did not seem correct because other acceptors such as oxygen (39) did not increase the yield of succinic acid; likewise increased yields of the reduced compound propyl alcohol were not accompanied by an increase in succinic acid.

The fact that Elsdén (40) had shown that the rate of succinic acid formation by *Escherichia coli* is a function of the concentration of carbon dioxide in the medium, further supported the role of carbon dioxide in succinic acid synthesis, and indicated that the phenomenon was not limited to the propionic acid bacteria.

Further investigations (41, 5) on the propionic acid fermentation did not bring forth much additional information as to the actual mechanism of the fixation. Carbon dioxide fixation was demonstrated with several substrates, and such inhibitors as malonate, azide, arsenite, cyanide and pyrophosphate were found to have no effect on the fixation, whereas sodium fluoride and iodoacetate did. The inhibition by fluoride caused an equivalent decrease in succinic acid formation in accordance with the concept of C_3 and C_1 addition. Some indication was obtained that phosphate had a function in carbon dioxide fixation.

The availability of carbon isotopes provided a great impetus to further investigation. Heretofore, studies on the mechanism of fixation were of necessity largely speculative, for although one could prove that carbon dioxide was fixed and actually entered into a carbon to carbon linkage, there was no method of determining its location in the carbon compound.

With isotopes the fixed carbon dioxide may be located in the compound. Work on the propionic acid fermentation with radioactive carbon was immediately initiated at the University of California and simultaneously a cooperative investigation between Iowa State College and the University of Minnesota was started with heavy carbon (C^{13}). Fixation by the propionic acid bacteria fermenting glycerol was confirmed with both the radioactive (16) and with heavy carbon dioxide (42, 14). The picture was not as simple, however, as previously thought, for the isotopic carbon was found in both the propionic acid and in the succinic acid in contrast to predictions made on the basis of the stoichiometric relationship between carbon dioxide fixed and succinic acid formed.

The fixation of carbon dioxide by coliform bacteria likewise was investigated by Wood *et al.* (42, 14), and with the C^{13} isotope a clear-cut fixation was demonstrated in the fermentation of galactose, pyruvate and citrate. Previous investigations, not using the isotope, had led to the proposal that these bacteria fix carbon dioxide but the results could not be conclusive, since there was always a net production of carbon dioxide in these fermentations. It is virtually impossible to prove conclusively, without isotopes, that carbon dioxide is both produced and assimilated in experiments involving a net increase in carbon dioxide, but with labelled carbon dioxide this can be accomplished. Fixation of carbon dioxide by the coliform bacteria occurred solely in the succinic and formic acids. The indications were, therefore, that all fixation in these fermentations was by C_3 and C_1 addition, except that formed by reduction of carbon dioxide to formic acid as described by Woods (25).

Nishina, Endo and Nakayama (43) by use of radioactive carbon have demonstrated the synthesis of malic acid and fumaric acid from pyruvic acid and carbon dioxide in fermentations by *Escherichia coli*. These authors prepared recrystallized derivatives of the acids; thus there is every reason to believe they were dealing with pure compounds. The results are significant because they demonstrate the presence of fixed carbon in compounds proposed to occur as intermediates in the conversion of oxalacetate to succinate. Radioactive fumarate also was demonstrated from a fermentation of glucose, and when ammonium chloride was added to the dissimilation of pyruvate, aspartate containing fixed carbon could be isolated. The scheme first proposed by Wood and Werkman (5) involving oxalacetate synthesis from pyruvate and carbon dioxide and a subsequent stepwise conversion to malate, fumarate and succinate, is suggested as the mechanism involved in these fixations. Krebs and Eggleston (44) have shown that this set of reversible reactions from oxalacetate to succinate occurs in

the propionic acid fermentation, demonstrating that such a set of reactions is feasible. No new data were supplied, however, on the actual fixation of carbon dioxide.

Other Possible Mechanisms of Succinate Formation.—Whether succinic acid is ever formed from carbohydrate by a mechanism other than fixation, *e. g.*, by acetic acid condensation or by decarboxylation of α -ketoglutaric acid as in the Krebs cycle, must await further investigation. The authors have presented evidence on several occasions which they believed to indicate a formation of succinic acid in the propionic acid fermentation by acetic or pyruvic acid condensation. The fact that fluoride had only a partial inhibitory effect on succinate formation from glucose, at concentrations which in glycerol fermentations almost completely inhibited the fixation of carbon dioxide as measured manometrically by the net decrease in carbon dioxide, led to this suggestion (5). These results together with other evidence made it appear that there was a fluoride insensitive mechanism of succinic acid formation which was independent of carbon fixation. This point has now been examined with heavy carbon dioxide, on the supposition that if there were such a mechanism, succinate formed in the presence of fluoride would not contain heavy carbon. The results (unpublished) show that fluoride does not prevent fixation of carbon dioxide in either the succinate or propionate formed from glucose by the propionic acid bacteria or in succinate formed from galactose, arabinose or mannose by *Escherichia coli*. This is apparent, for the C^{13} concentration was practically the same in the succinate from fermentations with or without addition of fluoride, and there was substantial fixation of carbon dioxide in each case. The yield of succinic acid per mole of substrate fermented was reduced in the presence of fluoride but apparently the succinate which was formed involved a fixation reaction. There was thus no indication by this method that succinate formation occurred by any other than the fixation reaction.

An explanation is now available for this incomplete inhibition of carbon dioxide fixation by fluoride. Studies with heavy carbon have shown that there is a formation of carbon dioxide from glycerol by propionic acid bacteria even in those experiments in which there is a net decrease in carbon dioxide in the system (14). This fact is apparent, for the C^{13} of the sodium bicarbonate and carbon dioxide was diluted during the fermentation. That the dilution was caused by $C^{12}O_2$ produced from the substrate is certain, inasmuch as the possibility of dilution by a miscellaneous exchange reaction was eliminated since all the C^{13} of the original system was accounted for in the products, residual sodium bicarbonate and carbon di-

oxide, at the conclusion of the experiment. Actually the carbon dioxide fixed in the glycerol fermentation by propionic acid bacteria is not just that observed manometrically as a net decrease in carbon dioxide but it is this quantity plus that produced from the glycerol by fermentation. The inhibition observed by fluoride was only that fraction of the total carbon fixation measured manometrically by a net decrease in gas. Thus the fixation in the glycerol fermentations was never completely inhibited by fluoride.

The carbon dioxide fixed in the presence of fluoride is almost entirely in propionic acid since there is little succinic acid formed under these conditions. There is reason to believe, however, that this carbon dioxide is initially fixed by C_3 and C_1 addition, and propionate is formed from the resulting C_4 dicarboxylic acid. This point will be considered later.

Recently Krebs and Eggleston (44) have rejected the idea that succinate may be formed by acetic acid condensation in the propionic acid fermentation. This reaction (or pyruvic acid condensation prior to formation of acetic acid) was proposed (45, 6) to account for low yields of acetic acid frequently found. Since in some cases the yield of succinate was not sufficient to account for the carbon dioxide formed on the basis that carbon dioxide equals the acetate plus twice the succinate, it was proposed that part of the succinate was in turn broken down to propionic acid and carbon dioxide. Krebs and Eggleston reject this explanation on the basis of the following points:

(a) They could not demonstrate an anaerobic breakdown of succinate and conclude that it is not metabolized by *Propionibacterium shermanii* anaerobically.

(b) They observed the simultaneous formation of both fumarate and succinate and state concerning this observation:

"Since the reaction, succinate \rightarrow fumarate, does not occur under anaerobic conditions, fumarate formed anaerobically cannot have arisen from succinate; if the formation of succinate by reduction is rejected, the improbable assumption of two separate mechanisms for the formation of succinate and fumarate must be made."

They suggest that the acetic acid is oxidized to carbon dioxide anaerobically by an unknown mechanism, and it is this reaction that causes the low yields of acetate and high yields of CO_2 .

The points raised in rejection of the Wood and Werkman scheme are not conclusive for the following reason. Shaw and Sherman (46), Hitchner (47), Wood *et al.* (6), and Fromageot and Bost (48) have all reported that succinate is fermented by propionic acid bacteria. In view of these findings

by so many different investigators, and extended over a period of eighteen years, it can be concluded that Krebs and Eggleston's conclusion that succinate is not fermented by *Propionibacterium shermanii* is incorrect. Erb (49) in this laboratory has studied the anaerobic breakdown of succinate by these bacteria (*Propionibacterium shermanii* included) rather extensively. He has found that washed cell suspensions from a five-day culture grown on a medium of yeast extract (Difco) 0.3 per cent, peptone (Difco) 0.2 per cent and glucose 1.0 per cent decarboxylate succinate anaerobically. The optimum pH for the reaction is 5.2; at pH 6.4 there was hardly any activity. The inactivity in Krebs and Eggleston's experiments may have been caused by the alkaline pH of the NaHCO_3 buffer.

Wood and Werkman (unpublished) have determined the products of this anaerobic breakdown of succinate. The rate of succinate fermentation falls off rapidly after the first six hours so that a large conversion was not obtained (15.7 mM per liter were fermented in two days by *Propionibacterium arabinosum*). After applying a correction for endogenous values (1.07 mM propionic acid, 1.36 mM acetic acid and 2.93 mM carbon dioxide), 15.84 mM propionic acid, 16.93 mM carbon dioxide and 3.6 mM acetic acid were obtained for the products. No other products were detected. The results show that the succinate was appreciably decarboxylated to propionic acid and carbon dioxide.

The experiments quoted by Krebs and Eggleston from Wood and Werkman (45) as direct evidence that succinate is not formed by condensation are satisfactorily explained, if one assumes a subsequent breakdown of succinate.

With regard to the second point three objections may be raised.

1. Under the proper experimental condition, *i. e.*, with methylene blue as an acceptor, the reaction succinate \rightarrow fumarate does occur. Whether it occurs in a natural dissimilation of glucose is a question that awaits further experimentation.

2. It is doubtful whether anyone would agree to rejection of the formation of succinate by reduction, for few would deny the possibility of succinate formation by carbon dioxide fixation yielding oxalacetate which is subsequently reduced to succinate.

3. Apparently a more crucial objection of Krebs and Eggleston to succinate formation by condensation is that this gives two mechanisms for formation of dicarboxylic acids, the second being by the fixation reaction. There is, however, a clear-cut example of the formation of succinate by two mechanisms. In the aerobic dissimilation of pyruvate by pigeon liver with addition of malonate as an inhibitor, succinate is

formed which contains no fixed heavy carbon, and in the same dissimilation fumarate is formed containing fixed heavy carbon (50). Obviously these two dicarboxylic acids arise by different reactions. Anaerobically in the absence of malonate, both succinate and fumarate are formed from pyruvate by pigeon liver. In this latter case, in which no inhibitor is added, there is every reason to believe that these acids arise by both the fixation reaction and a modified Krebs cycle. These reactions have not been shown to occur in the propionic acid fermentation but they certainly serve to illustrate that there is no reason *a priori* to conclude there cannot be two mechanisms. The authors have always held the view that propionic acid fermentation is more complex than generally believed, and that some modification is necessary in the scheme, be it inclusion of acetic acid condensation, pyruvic acid condensation or a modified Krebs cycle. The point is that Krebs and Eggleston have not furnished the necessary information to warrant rejection of any given proposal as yet—not that their proposals may not be correct. It may be of some interest in this connection to point out that the aerobic oxidation of propionic acid with liberation of carbon dioxide by propionic acid bacteria is more rapid than acetic acid oxidation.

Of some significance is the fact that in the large number of dissimilations studied with a variety of bacteria (*Propionibacterium*, *Escherichia*, *Aerobacter*, *Citrobacter*, *Proteus*, *Staphylococcus* and *Streptococcus paracitrovorus*) (14, 15) there has been fixation of carbon dioxide in succinate without exception, whenever it was formed. This indicates the general occurrence of the fixation reaction but does not necessarily eliminate the possibility that there are two mechanisms of formation of succinate. The fact that the amount of C^{13} fixed in succinate varies over a rather wide range in different experiments may be an indication that there is more than one source of succinate. The formation of succinate by a mechanism not involving C^{13} fixation would dilute the C^{13} of the succinate formed by fixation.

The Location of Fixed Carbon in Succinate.—There is good evidence that carbon dioxide is fixed by a C_3 and C_1 addition; however, assuming that fixation does occur by C_3 and C_1 addition, the elucidation of the actual mechanism is difficult even with the use of isotopes. The evidence that there is fixation by C_3 and C_1 addition will be considered first, and then preliminary studies will be discussed of what is believed to be the initial reaction of carbon dioxide fixation.

The formation of succinic acid, particularly, is believed to involve C_3 and C_1 addition. In this case the fixed carbon dioxide should be in the terminal carboxyl group of succinic acid. That this is the location of the fixed carbon has been definitely shown (51, 52) by degrading succinic acid contain-

ing fixed $C^{13}O_2$. Succinic acid isolated from fermentations by *Propionibacterium pentosaceum*, *Escherichia coli*, *Proteus vulgaris*, *Aerobacter indologenes* and *Streptococcus paracitrovorus* has been tested, and in each the methylene carbon atoms have been found to contain the normal complement of C^{13} , whereas the carboxyl groups contain all the fixed heavy carbon. The succinic acid was decarboxylated (51) by converting it to a mixture of fumaric and malic acids with a heart muscle preparation containing succinic dehydrogenase and fumarase. The malic acid was oxidized to two molecules of carbon dioxide and one of acetaldehyde by acid permanganate. The aldehyde is from the methylene groups and the carbon dioxide from the carboxyl groups.

Only by quantitative methods is it possible to prove that a symmetrical dicarboxylic acid contains fixed carbon in both its carboxyl groups. If the concentration of C^{13} in the carboxyl groups is greater than the average value calculated on the basis that one carboxyl group has a normal complement of C^{13} and the other a complement equivalent to that of the $C^{13}O_2$ available for fixation, obviously fixation has occurred in both carboxyl groups of at least part of the dicarboxylic acid molecules. If the C^{13} concentration in the carboxyl groups is less than or equal to this average value there is no means available at present to determine whether any one molecule has more than one carboxyl containing fixed carbon. In none of the fermentations so far examined (51, 52) has there been a concentration of C^{13} in the carboxyl carbons of the succinate sufficiently high to prove fixation in both carboxyl groups. In the propionic acid fermentation of glycerol the concentration of C^{13} in the carboxyl groups of succinate approaches rather closely the value estimated for fixation in only one carboxyl group. This fact may be interpreted as evidence that succinate is formed in this fermentation solely by C_3 and C_1 addition, the C_3 compound being formed from the glycerol with little or no exchange of its carbon with $C^{13}O_2$.

The Initial Reaction in C_3 and C_1 Addition.—As a working hypothesis the reaction $CO_2 + CH_3 \cdot CO \cdot COOH \rightleftharpoons COOH \cdot CH_2 \cdot CO \cdot COOH$ was proposed (4, 42) as the possible initial conversion in fixation of carbon dioxide by C_3 and C_1 addition. It should be clearly understood that at best the reaction represents merely the over-all conversion. It is quite possible and even probable that phosphorylated intermediates are involved and that the reaction is more complex than represented. The proposal that the fixation of carbon dioxide by C_3 and C_1 addition is a reaction of general biochemical importance has been advanced by the authors for the past several years (4) without general acceptance until recently. The present tendency is, perhaps, to the other extreme in that suggestions involving

some speculation may now be too readily accepted. This results, in part, from the great general interest in isotopic investigations and a consequent focussing of attention on studies on fixation of carbon dioxide. The fixation reaction has recently been suggested by Solomon, *et al.* (53), as a part of a possible mechanism of fixation of carbon dioxide in glycogen synthesized from lactate by liver; also Meyerhof (54) has used the reaction to explain the Pasteur effect. These authors are fully aware that they are dealing with complex phenomena for which a number of possible explanations may be offered, and, furthermore, that since the mechanism of the fixation reaction has not been fully elucidated, there can be no certainty as to how it fits into a scheme. It is desirable, however, that such ideas be advanced as working hypotheses. Inasmuch as the fixation reaction is used frequently in a wide variety of schemes, there is a tendency to accept the reaction as fact and to forget that the actual mechanism of the reaction is not completely established.

Krebs and Eggleston (55, 44) in two articles entitled, "The Biological Synthesis of Oxalacetate from Pyruvic Acid and Carbon Dioxide," imply that they show carbon dioxide fixation with pyruvic acid to form oxalacetate. In no case, however, was a synthesis of oxalacetate shown; furthermore, fixation of carbon dioxide was not demonstrated and in one of the investigations pyruvic acid was not used as a substrate. Their principal experimental contribution, in so far as carbon dioxide fixation is concerned, was to show that an increased carbon dioxide concentration stimulates succinate formation from pyruvate by pigeon liver. In addition they provided evidence that cocarboxylase has a function in fixation (*cf.* page 158).

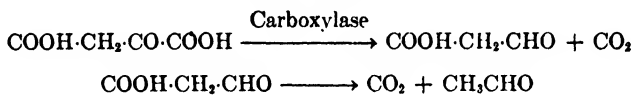
The present status of evidence relative to the actual mechanism of the fixation reaction is outlined below. It should be emphasized that the uncertainty as to the mechanism of C_3 and C_1 addition in no way alters the evidence (51, 52) that there is such a fixation. The question is not so much whether it occurs, but how it occurs. -Krebs' (56) statement:

"This (Evans and Slotin's demonstration of fixed carbon in α -ketoglutarate) completes the proof of the occurrence of reaction (9) ($\text{COOH}\cdot\text{CO}\cdot\text{CH}_3 + \text{CO}_2 = \text{COOH}\cdot\text{CO}\cdot\text{CH}_2\cdot\text{COOH}$) in pigeon liver,"

is hardly accurate. The position of the fixed carbon in α -ketoglutarate was not known at this time, and even now that the position of the fixed carbon is known, it can only be said that the results do not conflict with the proposed mechanism of fixation. Fixation in the carboxyl group of fumarate or malate, for example, will give the same result as fixation in oxalacetate

since these compounds are convertible to oxalacetate. For that matter, it has not been proved that oxalacetate, as such, is the compound that reacts with pyruvic acid in the Krebs' cycle. The only way to prove that carbon dioxide can be fixed by addition to pyruvic acid is to use isolated enzyme systems and to isolate the oxalacetic acid under acceptable conditions. The enzyme system must be such as to eliminate miscellaneous reactions which confuse the picture. Even with evidence such as this, objection can be raised that the fixation was not conducted under natural conditions.

The fixation reaction as represented in the above equation is a carboxylation of pyruvic acid and implies that the decarboxylation of oxalacetic acid is reversible. Until recently the only enzymes known to decarboxylate oxalacetic acid were carboxylase and the thermostable enzyme from muscle studied by Breusch (57). There is no certainty that carboxylase acts on the carboxyl next to the methylene carbon in oxalacetate. Its action may well be on the carboxyl next to the carbonyl to yield malonic aldehyde, which breaks down spontaneously to acetaldehyde and carbon dioxide.



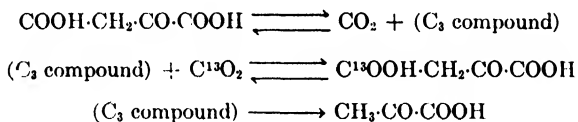
In this case carboxylase would not be a component of the fixation reaction since it activates the carboxyl adjacent to the carbonyl group instead of the one adjacent to the methylene group. The thermostable enzyme studied by Breusch has a very low activity and is probably not concerned in the fixation reaction.

Krampitz and Werkman (58) have recently discovered a new enzyme which offers promise of being one involved in the fixation reaction. The enzyme is heat-labile and catalyzes the decarboxylation of oxalacetate to pyruvate. An acetone and alkaline phosphate washed preparation of *Micrococcus lysodeikticus* is used to demonstrate the presence of the enzyme. Such preparations are free from cocarboxylase and Mg^{++} and will not decarboxylate or oxidize either oxalacetate or pyruvate. When Mg^{++} is added, the enzyme decarboxylates the oxalacetate, and there is no further action on the resulting pyruvate. When cocarboxylase is then added, the resulting pyruvate is oxidized to acetic acid and carbon dioxide. It is therefore evident that the decarboxylation of oxalacetate by this enzyme is dependent on Mg^{++} but independent of cocarboxylase.

On the basis that this enzymic reaction is reversible, and that the equilibrium is not too far to the side of pyruvic acid, the synthesis of oxalacetate from pyruvate and carbon dioxide should be possible with this enzyme.

Krampitz and his co-workers (59) attempted this synthesis but were unable to demonstrate any formation of oxalacetate. With $C^{13}O_2$ they have obtained evidence, however, that the enzyme is involved in the carboxylation or fixation reaction.

A decarboxylation of oxalacetic acid was conducted in the presence of $C^{13}O_2$ with the deficient preparation to which Mg^{++} was added. The decarboxylation was allowed to proceed until it was approximately 50 per cent complete, and then the C^{13} concentration in the carboxyl adjacent to the methylene group was determined in the residual oxalacetate. The necessary carboxyl was obtained by decarboxylation with citric acid and aniline (60). The resulting carbon dioxide contained a concentration of C^{13} (1.4 per cent) significantly above the normal. On the contrary, similar experiments conducted without addition of the enzyme contained substantially no fixed carbon. Clearly the enzyme catalyzed the induction or exchange of carbon dioxide with the carboxyl group of oxalacetate. Essentially this exchange is C_3 and C_1 addition, for the products of the enzymic conversion are solely C_3 and C_1 compounds and there is no reason to believe the intermediate steps involve other carbon chains. The following reactions are proposed as a possible explanation of the observed exchange during decarboxylation of oxalacetate and the failure to obtain a synthesis of oxalacetic acid from pyruvic acid and carbon dioxide.



It is suggested that the C_3 compound in parentheses is a derivative of pyruvic acid, possibly a phosphorylated compound, and that it is this compound rather than pyruvic acid that is a component of the fixation reaction. In addition it is suggested that the unknown C_3 compound is converted to pyruvic acid, and that in the system as employed by Krampitz *et al.*, this reaction is not reversible. Pyruvic acid thus would not be suitable for demonstration of the synthesis of oxalacetic acid. Furthermore, the decarboxylation of oxalacetic acid may not be by a direct splitting out of carbon dioxide but may involve a preliminary phosphorylation. Additional studies are necessary before these possibilities can be evaluated fully. Nevertheless, the above results offer the first direct evidence that oxalacetic acid is a component in the fixation reaction. No previous investigation has shown a fixation in oxalacetic acid, assumed to be the initial product of C_3 and C_1 addition.

The question arises whether or not an exchange of carbon dioxide with the carboxyl group occurs during the action of carboxylases in general or is specific for the particular carboxylase which acts on the carboxyl beta to the keto group of oxalacetic acid. If there is a similar exchange with other carboxylases, the possibility exists of fixation of carbon dioxide by a rather large number of reactions, and the theory that C_2 and C_1 addition is the principal path for fixation of carbon dioxide by typical heterotrophs would probably have to be revised. The evidence for fixation of carbon dioxide by other mechanisms will be considered in a later section, but usually such mechanisms have not been found in typical heterotrophs. It is true that some exchange might be expected when any carboxylase is active but for most carboxylases the equilibrium may be so far to the side of decarboxylation that from a practical standpoint the reaction is irreversible. The extent of exchange of carbon dioxide with the carboxyls of pyruvic, lactic and α -ketoglutaric acids during aerobic decarboxylation by *Micrococcus lysodeikticus* has been determined by Krampitz *et al.* (59). The decarboxylation of the acids was allowed to proceed in the presence of $C^{13}O_2$ until somewhat over 50 per cent of the acid was converted, then the C^{13} concentration in the carboxyl group was measured. The results showed that there was practically no exchange in the carboxyl group of these acids under the conditions of the experiments. Pyruvate arising from lactate was, likewise, tested with negative results. The absence of exchange in this pyruvate is, perhaps, of greater significance than in the direct use of pyruvate since it was formed within the cell and in all probability came in contact with the active enzyme centers, whereas there is no assurance that such are the conditions when starting with pyruvate and determining C^{13} in the unfermented portion. More extensive studies are needed before a definite conclusion can be drawn, but at present the possibility of a general fixation of carbon dioxide through the action of carboxylases as a group seems remote. Fixation by carboxylases may be limited largely to the enzyme studied by Krampitz *et al.* Evans (61), likewise, has found no evidence of exchange during the action of carboxylase on pyruvate. The exchange was studied by use of radioactive carbon dioxide at pressures as high as 300 atmospheres. Ruben and Kamen (17) also consider the reaction irreversible.* Their evidence is hardly conclusive. Experiments were conducted in which yeast suspensions, with no added substrate, fixed radioactive carbon dioxide. By use of pyruvate as a carrier, a fraction was isolated as the hydrazone. This hydrazone contained but a small per cent of the total fixed radioactive carbon. There was no proof provided, however, that there actually was

* However, cf. *Proc. Natl. Acad. Sci. U. S.*, 27, 475 (1941).

any pyruvate formed in the dissimilation (none was added apparently). The negative result, therefore, could have been caused either by the absence of an exchange or because there was not enough pyruvate present to give a detectable amount of fixed carbon, even though an exchange did occur.

Proof that there is no exchange in pyruvate is of considerable importance because, both in bacterial fermentations (15) and in the dissimilation of pyruvate by animal tissue (50), lactate is formed containing fixed carbon in the carboxyl group. Since pyruvate is generally believed to be the precursor of lactate, fixation by exchange in the pyruvate is one of the possible mechanisms of fixation in lactate. Other mechanisms for this fixation will be considered later.

Relationship of Cocarboxylase to the Fixation Reaction.—In contrast to the findings of Krampitz and Werkman (58) and Krampitz *et al.* (59), Krebs and Eggleston (55) and Smyth (62) believe that cocarboxylase is essential for the fixation of carbon dioxide in oxalacetate. Krebs and Eggleston (55), reasoning from indirect evidence with pigeon liver and from analogy with bacterial fixation of carbon dioxide (5), concluded that there is a synthesis of oxalacetic acid from pyruvic acid and carbon dioxide in pigeon liver. The evidence will be considered in greater detail in the section on animal fixation of carbon dioxide, but it is entirely indirect since a net uptake of carbon dioxide was not demonstrated. The proposed function of thiamin in the fixation reaction is based on the observation that thiamin, on addition to muscle and liver suspensions from thiamin-deficient pigeons, causes an increased dissimilation of pyruvate by liver but not by muscle. The vitamin, therefore, was presumed to take part in a reaction present in liver but absent in muscle. The fixation reaction was believed by Krebs and Eggleston to meet this requirement, and they, therefore, concluded that the vitamin acts in this fixation reaction. Evans and Slotin (63) have since presented evidence that there is, in fact, no fixation of carbon dioxide by pigeon breast muscle. The action of carboxylase in pyruvate oxidation, according to Krebs and Eggleston's concept, is to synthesize oxalacetate which is necessary as a hydrogen carrier and as a component of the Krebs cycle. The stimulating action of cocarboxylase in apparently dissimilar reactions, *i. e.*, the non-oxidative decarboxylation of pyruvate by yeast and oxidative decarboxylation by animal tissue and bacteria, was believed to be explained on the basis that decarboxylation of pyruvate and carboxylation of oxalacetate were enough alike so that they would be catalyzed by similar or identical enzymes, both requiring cocarboxylase. The usual assumption of a dual function of a carboxylase as a catalyst was thus avoided.

Although the above explanation of an analogous function of carboxylase in oxidative and non-oxidative decarboxylation of pyruvate may seem attractive, there are several facts that argue against these proposals. The experimental evidence presented by Krebs and Eggleston in support of the proposed function of cocarboxylase is hardly conclusive. They presented no evidence to show that the breast muscle preparation was actually deficient in cocarboxylase. If such were not the case, addition of cocarboxylase would not increase the rate of pyruvate oxidation by the muscle. Ochoa and Peters (64) have shown that there is a marked difference in the vitamin content of the different organs of an animal showing effects of vitamin deficiency, and that liver usually has a lower content of the vitamin than the muscle. The observed difference in the effect of the vitamin on addition to muscle and liver may not have resulted from a stimulation of a reaction present in liver and absent in muscle but, instead, because the liver was deficient in cocarboxylase and the muscle was not. It also has been pointed out by Barron (65) that the difference may have resulted because minced muscle is unable to phosphorylate thiamin rapidly (66). The necessary cocarboxylase, in this case, would not be formed in muscle, but would be formed in liver.

Aside from these weaknesses in experimental proof, and the evidence of Krampitz and Werkman that cocarboxylase is not necessary in the reaction in the case of *Micrococcus lysodeikticus*, the concept is not convincing, for it is not in agreement with known facts of pyruvate oxidation. Banga and her co-workers (67) have shown that besides cocarboxylase, a C₄ dicarboxylic acid is an essential component of the pyruvate oxidizing system. Clearly in these oxidations, the function of the carboxylase is not to synthesize C₄ dicarboxylic acids as proposed by Krebs and Eggleston.

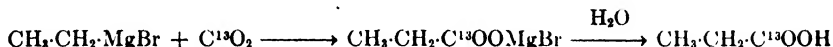
Smyth's evidence (62) that thiamin is a component of the fixation reaction was obtained from a study of the dismutation of pyruvate by thiamin deficient *Staphylococcus*. The dismutation could be stimulated either by the addition of thiamin or oxalacetate. In accordance with the Krebs and Eggleston concept, it was proposed that the function of the thiamin in the pyruvate dismutation was to promote synthesis of oxalacetate which is necessary as a hydrogen carrier. When oxalacetate is present, the need of thiamin, therefore, is removed. At present there seems to be no other ready explanation of Smyth's results. The system is complex, however, and it is by no means certain that Smyth's explanation is correct. Krebs and Eggleston (44) have reported recently that addition of oxalacetate, fumarate or malate catalytically accelerated the fermentation of glycerol by propionic acid bacteria. There is no reason to believe the fixation reac-

tion was impaired in any way in these fermentations, yet oxalacetate stimulated the dissimilation. This serves to illustrate that stimulation with oxalacetate as observed by Smyth may not have resulted because of a weak fixation reaction, but for some other reason, as in the propionic fermentation. At any rate it can be concluded that the above-proposed action is not the function of cocarboxylase in the dissimilation of pyruvate by *Micrococcus lysodeikticus* (58) for in this case both Mg^{++} and cocarboxylase are necessary for the oxidation of pyruvate even when oxalacetate is added.

On the basis of present results it is apparent that there is fairly direct evidence (58) that cocarboxylase is not a component of the fixation reaction as such. It is conceivable that in the complex reactions studied by Krebs and Eggleston and by Smyth there may be an indirect connection between carboxylase activity and carbon dioxide fixation, but if there is, the mechanism is obscure at present.

The Location of Fixed Carbon in Propionic Acid.—The only compound other than C_4 dicarboxylic acids that definitely has been proposed to arise by C_3 and C_1 addition is propionic acid. Carson and Ruben (16) and Wood *et al.* (42, 14), independently with isotopic carbon found that propionic acid formed in the fermentation of glycerol by *Propionibacterium* contains fixed carbon dioxide. Both proposed that the carbon is initially fixed by C_3 and C_1 addition and that the propionate arises from a dicarboxylic acid. Accordingly, the fixed carbon would be in the carboxyl group. Carson and his co-workers (68), degraded propionate obtained from the fermentation of glycerol, and erroneously concluded that the fixed carbon is located not only in the carboxyl group but is probably equally distributed among all three carbon atoms of the molecule. Wood *et al.* (69), then isolated propionic acid containing fixed C^{13} from a fermentation of glycerol and degraded the acid by a different set of reactions. They found the fixed carbon to be exclusively in the carboxyl group. The degradation was accomplished by α -bromination to give bromo-propionic acid, conversion of this compound to lactate with silver hydroxide, and oxidation of the lactic acid with acid permanganate to acetaldehyde and carbon dioxide. The carbon dioxide is formed from the carboxyl group of the propionate, the acetaldehyde from the α - and β -carbon atoms. The aldehyde contained a normal per cent of C^{13} , and the carbon dioxide a high per cent, *i. e.*, all the fixed carbon. In order to make certain that the conflicting observations were not the result of a difference in the synthetic reactions of the bacteria under the respective experimental conditions, the reliability of the reactions used by Carson *et*

al., was determined with propionic acid synthesized by the following reaction:



The resulting acid was degraded by the reactions used by Carson *et al.* (68), *i. e.*, alkaline permanganate oxidation and dry distillation of the barium salt. Oxalate and carbonate are formed in the permanganate oxidation. It was assumed by Carson *et al.*, that the carbonate arose from the carboxyl group and oxalate from the α - and β -carbon atoms. The results (70) from the degradation of the synthetic acid showed definitely that the reaction does not occur as assumed, for the carboxyl carbon was found in both the oxalate and carbonate. In the dry distillation of barium propionate, diethyl ketone and carbonate are formed. According to the accepted mechanism of the reaction 50 per cent of the carboxyl carbon should be in the carbonate, the other 50 per cent in the ketone. This distribution of carboxyl carbon was found with the synthetic acid (70). Carson *et al.* (71), and Nahinsky and Ruben (72) independently have re-investigated the problem. They likewise have found with synthetic propionic acid containing C^{14} radioactive carbon in the carboxyl group, that the alkaline permanganate oxidation is not reliable. The dry distillation of the barium salt apparently was not checked with the synthetic acid to determine the reliability of this degradation. Judging from the results of Wood *et al.* (70), however, it seems probable that the reaction is reliable, and a faulty experimental procedure was used in the original experiments of Carson *et al.* At any rate Carson and his co-workers (71) on reinvestigation with the biological acid have found the fixed carbon only in the carboxyl group.

This series of investigations serves to illustrate the difficulty faced in determining the location of isotopic carbon in a compound, and also in synthesizing compounds which will contain isotopic carbon in certain positions. The exact mechanism of the chemical reactions which are employed are often not known. Under the circumstances it is necessary to study the mechanism of the reaction before use. Much experimental work is needed in this field before the full benefits of isotopes can be realized.

The Mechanism of Fixation of Carbon Dioxide in Propionic Acid.—With the position of the fixed carbon definitely shown to be in the carboxyl group of the propionate, the problem of the mechanism of fixation can be considered more accurately. The following mechanism has been proposed by Carson, *et al.* (71), and Krebs and Eggleston (44) for formation of propionate containing fixed carbon:

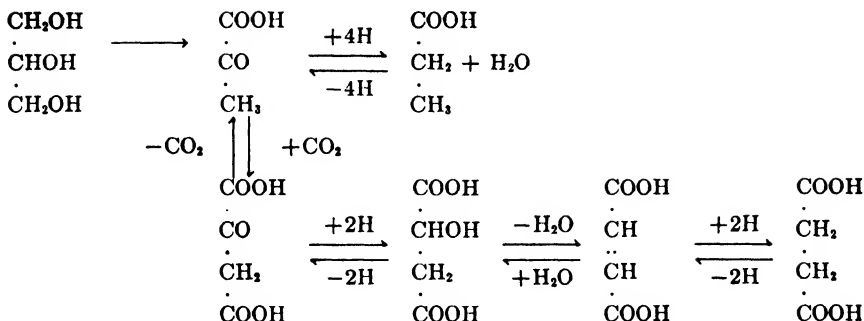


Fig. 2.

Viewed critically there is not a great deal of evidence to support the scheme, other than the fact that it provides a mechanism for the fixation of carbon dioxide in the carboxyl group of propionic acid. Krebs and Eggleston (44) have shown that the reactions from oxalacetic acid to succinic acid are reversible with the propionic acid bacteria. It has not been shown conclusively, however, that under the conditions of a glycerol fermentation a significant amount of fumarate or succinate is converted to propionate. According to the scheme (Fig. 2) it is essential that the reaction proceed as far as a symmetrical molecule (fumarate or succinate) and then reverse itself so that pyruvate may be formed containing fixed carbon. It is questionable whether much fumarate would be oxidized to oxalacetate in the presence of glycerol which is a good hydrogen donor.

If there was a rapid shifting back and forth from pyruvate to fumarate, part of the dicarboxylic acids should contain fixed carbon in both carboxyl groups, since some pyruvic acid containing fixed carbon would be present and could re-enter the fixation reaction. According to the scheme those dicarboxylic acids not containing two fixed carbons would contain at least one, therefore the average should be well above fixation of one carbon. Actually the quantitative data of Wood *et al.* (14), indicate that the succinate formed in the glycerol fermentation contains only one fixed carbon atom. This fact is a strong argument against any scheme that implies a part of the succinate is to contain two fixed carbons. The evidence supporting the contention that there is approximately only one fixed carbon in each succinate molecule has been arrived at by the following calculation. It has been assumed that the concentration of C^{13} in the carbon dioxide fixed by the cell is equal to that of the medium at the conclusion of the fermentation. It is difficult to estimate the concentration of C^{13} in the carbon dioxide available to the cell, since there is no assurance that the

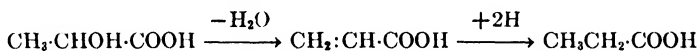
carbon dioxide produced within the cell comes to equilibrium with that dissolved in the medium. The above assumption probably gives a minimal value for the available $C^{13}O_2$, for at the start of the experiment the C^{13} concentration is higher than at the conclusion when dilution with $C^{12}O_2$ from the substrate has occurred. At any rate, on this basis and the assumption that only one $C^{13}O_2$ is fixed in each succinate molecule, the calculated values for the C^{13} in the succinate of two experiments are 1.72 and 1.34 per cent. The observed values were 1.65 and 1.28 per cent, respectively. Calculated on the basis that both carboxyls contain fixed carbon, the values are 2.71 and 1.77 per cent. The calculations are approximations but, nevertheless, they seem to indicate that fixation occurs in only one carboxyl group.

Accordingly, the scheme in Fig. 2 involves the splitting of a carboxyl from a symmetrical dicarboxylic acid containing fixed carbon in only one position to form propionate containing fixed carbon. Wood and his co-workers (14) have presented quantitative evidence to show that practically every molecule of propionate formed from glycerol would have to pass through fumarate if the above scheme holds. If every molecule of propionate was formed *via* decarboxylation of a symmetrical dicarboxylic acid and none directly from the glycerol, the C^{13} concentrations in the carboxyl group of the succinic and propionic acids should be equal. In the two fermentations so far examined the values were 2.21 and 1.47 per cent C^{13} for the carboxyl of propionic acid as compared to 2.29 and 1.54 per cent, respectively, for the carboxyls of succinate. The implication is that none of the propionate formed in the fermentation of glycerol has arisen by direct reduction of pyruvate prior to its conversion to fumarate and back again. This argument holds whether it is accepted that succinate contains only one fixed carbon or not. It seems unlikely on the basis of Fig. 2 that not any of the propionate will be formed by reduction of pyruvate before it passes through fumarate and back again. Furthermore, it is unlikely that once the propionate is formed there will be any extensive passage back to pyruvate. There is every reason to believe that if there is an equilibrium between pyruvate and propionate, it is far to the side of propionate, for in a propionic acid fermentation, pyruvate can be detected only by special methods.

Carson and his co-workers (71) have offered evidence which they suggest may show that there is interconversion of propionate and succinate. They admit that the rates of interconversion may be too slow to account for the radioactivity usually found in propionic acid formed from glycerol. In fact, on addition of radioactive succinate (obtained from the propionic fer-

mentation) to glycerol or pyruvate fermentations containing no other source of C^{11} , the radioactivity of the formed propionate was found to be 2 ± 2 and 1 ± 1 units, respectively, on the addition of 100 units of succinate. In the case of added propionate the recovered succinate was 5 ± 2 and 9 ± 2 units. Even if these figures are considered significant, there is no assurance that the succinate and propionate are formed by the reversible series of reactions of Fig. 2. There is a possibility that the succinate may be decarboxylated directly to propionate; also that the propionate is oxidized with liberation of radioactive carbon dioxide which then is fixed in the succinate. In one fermentation in which radioactive propionate was added 8 ± 1 units of carbon dioxide were formed.

Most of the above discussion relative to the mechanism of formation of propionate has been of a negative type, in that it has pointed out only the weaknesses of existing scheme of Fig. 2. What constructive suggestions can be offered? Frankly, sufficient data are not available with which to formulate a defensible scheme. It is likely that the mechanism of succinate formation from glycerol by the propionic acid bacteria is substantially as shown in the above scheme with the exception that in the presence of glycerol the series of equilibria between pyruvate and succinate are largely shifted to succinate, owing to the reducing intensity of the glycerol. This would account for the succinate containing only one fixed carbon per molecule, a conception which has been our basic assumption since 1938. Elucidation of the mechanism of propionic acid formation has always been a difficult problem. Of several mechanisms proposed, that involving removal of water from lactic acid to form acrylic acid which is then reduced to propionic acid has seemed the most probable.



Up to the present, however, the reduction of acrylic acid by propionic acid bacteria has not been demonstrated. As shown in the previous discussion, there is some basis for assuming that all the propionic acid formed in the fermentation of glycerol may arise by decarboxylation of a symmetrical dicarboxylic acid containing one fixed carbon. Accordingly, 50 mM of $C^{12}\text{O}_2$ will be formed for each 100 mM of propionate, since there is an equal chance of splitting out the carboxyl of the dicarboxylic acid which originates from the glycerol and the carboxyl that is formed by fixation of $C^{13}\text{O}_2$. If the C^{13} of the sodium bicarbonate be calculated on the basis of this dilution by $C^{12}\text{O}_2$, there is reasonable agreement between the experimentally observed and calculated values (calculated: 3.76 and 2.23; observed: 3.62 and 2.10 per cent C^{13}). This is further evidence that the propionic acid is

formed in the glycerol fermentations exclusively by decarboxylation of a symmetrical dicarboxylic acid. It is the mechanism of the decarboxylation that particularly is uncertain. The evidence that the mechanism does not involve the reversible reactions of Fig. 2 has already been discussed. There is some evidence that the propionic acid bacteria can decarboxylate succinate anaerobically ($\text{COOH}\cdot\text{CH}_2\text{CH}_2\cdot\text{COOH} \rightarrow \text{CO}_2 + \text{CH}_3\text{CH}_2\cdot\text{COOH}$) but it is questionable whether or not the rate of this reaction is high enough to be of any considerable importance.

Whether or not propionate may be formed from glucose and pyruvate exclusively by the fixation reaction, *i. e.*, by C_3 and C_1 addition with subsequent decarboxylation of a dicarboxylic acid is uncertain, and there is some evidence to the contrary. Carson and his co-workers (71) in the fermentation of pyruvic acid by the propionic acid bacteria found 5 per cent of the fixed radioactive carbon in the volatile acids and 95 per cent in the non-volatile acid. Since the yield of volatile acids, and especially of propionic acid, was not given, no definite idea can be reached as to how much of the total propionic acid was formed by the fixation reaction. If the yield of volatile acid was at all normal, however, and the propionic acid was formed through the fixation reaction, there would have been more fixed carbon in the volatile acid fraction than was observed.

In a preliminary unpublished experiment by the authors, it has been found, contrary to the results of Carson *et al.*, that a large part of the fixed carbon is in the volatile acid from a pyruvate fermentation. However, by the same method of calculation that was employed in the glycerol fermentation, only 60 per cent of the propionic acid is indicated to have arisen by the decarboxylation of a symmetrical dicarboxylic acid. In this calculation it is assumed that all the C_4 dicarboxylic acids are formed by the fixation reaction. In the fermentation of glucose and pyruvate there is some indication that more than one mechanism of succinate formation occurs. Therefore, there is a possibility that all the propionate is formed by decarboxylation of a symmetrical dicarboxylic acid.

The experiment by Carson *et al.* (71), was of short duration (50 minutes), whereas that of the authors ran for 18 hours, *i. e.*, until the pyruvate was all fermented. This experimental difference may account for the discrepancy in the amount of fixed carbon in the volatile acids. Carson and his co-workers also reported the formation of a non-volatile keto acid which contained 70 per cent of the fixed carbon of the non-volatile acid fraction. The acid was not pyruvic acid and was not identified. Its identification is of interest because of the light that might be thrown on the mechanism of carbon dioxide fixation. The compound can hardly be oxalacetic acid, for

this acid is rapidly decomposed on heating in acid solution and very likely would have been converted to pyruvate during the steam distillation.

It is evident from the above discussion that propionic acid is formed by a fixation reaction and probably by decarboxylation of a C_4 dicarboxylic acid that is formed by C_3 and C_1 addition. The mechanism of decarboxylation is not known, neither is it known whether or not this reaction is the general mechanism by which propionate is formed in all propionic acid fermentations.

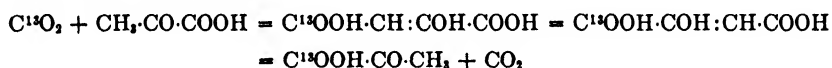
2. Miscellaneous Fixation Reactions

There have been a number of other fixation reactions demonstrated, but information is as yet too meager to allow any definite idea of their mechanisms. In some cases it is probable that the mechanism is simply the cleavage of a C_4 compound in which carbon dioxide has been fixed by C_3 and C_1 addition. The mechanism, thus, is fundamentally the same as described under C_3 and C_1 addition, in so far as the fixation itself is concerned. In others it is evident that the fixation reactions may involve the formation of a carbon chain entirely from C_1 compounds. Particularly this latter type of synthesis is not well understood. It is here that information is needed to solve the mechanism of the strict autotrophs and of photosynthesis.

Examples of fixations that may occur by C_3 and C_1 addition or, on the other hand, may be proved to involve a mechanism quite different, have recently been demonstrated by Slade *et al.* (15, 52). They have investigated fixation of carbon dioxide by several of the typical heterotrophic bacteria through use of $C^{13}O_2$. The compounds found in the different fermentations have been isolated and their C^{13} content determined. The species used were *Staphylococcus candidus*, *Aerobacter indologenes*, *Streptococcus paracitrovorus*, *Clostridium welchii*, *Clostridium acetobutylicum*, *Proteus vulgaris*, *Lactobacillus plantarum* and *Streptococcus lactis*. Glucose and citrate were used as substrates with cell suspensions in most cases. The last two species are homo-lactic acid bacteria, *i. e.*, bacteria that form substantially nothing but lactic acid. There was no evidence of fixation by either of these bacteria but there was fixation by all the other cultures. It has been pointed out previously that all the genera forming succinate (*Staphylococcus*, *Aerobacter*, *Streptococcus* and *Proteus*) fixed carbon in this compound.

Fixation in Lactate and Acetate.—Of particular interest is the observed fixation in lactic acid and acetic acid (15, 52). There was fixation in lactic acid by the following species: *Staphylococcus candidus*, *Streptococcus para-*

citrovorus, *Clostridium welchii*, *Clostridium acetobutylicum*, *Proteus vulgaris* and *Aerobacter indologenes*. The carbon fixed in the lactic acid has been found in each case to be exclusively in the carboxyl group as located by acid permanganate oxidation of the lactic acid to carbon dioxide and acetaldehyde. The carbon dioxide arises from the carboxyl carbon, the acetaldehyde from the α - and β -carbons. The fact that the homo-lactics do not fix carbon dioxide, as do the hetero-lactics, may be of some significance in indicating that a cleavage of the C_3 chain is necessary before fixation of carbon dioxide in lactate can occur. On the other hand, the failure of the homo-lactic acid bacteria to fix carbon dioxide in lactic acid may result from an inability to fix carbon dioxide by a direct C_3 and C_1 addition. A possible mechanism of fixation of carbon dioxide in lactic acid by C_3 and C_1 addition is as follows:



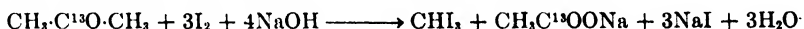
Pyruvic acid containing heavy carbon is formed in this reaction by shifting the hydroxyl and hydrogen of enol-oxalacetic acid and subsequently decarboxylation. Meyerhof (54) has proposed this reaction and suggests that the shifting of hydroxyl with hydrogen occurs spontaneously. The formation of pyruvate containing fixed carbon thus can be explained without passage through a symmetrical C_4 dicarboxylic acid. The pyruvate is then reduced to lactate. A similar scheme might apply to the formation of propionate. The same objection may be raised to this scheme as with others, *i. e.*, if heavy carbon pyruvate is present, part of the dicarboxylic acids should contain two fixed carbons. In no case has the content of fixed carbon in succinate been sufficient to indicate such an occurrence. This may be due, however, to the simultaneous formation of succinate by a non-fixation reaction.

It is significant that, in contrast to the fermentation of glucose, the fermentation of citrate by *Streptococcus paracitrovorus* yields succinate which contains fixed carbon and lactate which does not contain it. This fact may indicate that carbon is not fixed in lactate by the above equilibria for if such were the case, it would be expected that whenever C_4 dicarboxylic acids were formed containing heavy carbon, heavy carbon pyruvate and lactate would likewise occur. It is apparent that more information is needed before a decision can be reached on the mechanism of fixation of carbon in lactate. The possibility must be left open that heavy carbon lactate is formed by C_2 and C_1 addition but there is no evidence to support this idea at present.

Acetic acid containing fixed carbon was formed by *Aerobacter indologenes* and by *Clostridium welchii*. The fixed carbon has been shown to be exclusively in the carboxyl group. The procedure of degradation (*cf.* Barker *et al.* (18)) involved dry distillation of the barium salt to yield acetone and barium carbonate.



The acetone was then degraded by the iodoform reaction.



The barium carbonate contained heavy carbon, whereas there was none in the iodoform. The mechanism of this fixation is unknown. It is possible that the acetic acid is formed by cleavage of a C_4 dicarboxylic acid. No detectable amount of C_4 dicarboxylic acid was formed in the fermentation by *Clostridium welchii* but this does not necessarily mean that it did not occur as an intermediate. Further studies are being made on the mechanism of this fixation, for it offers possibilities of representing a type differing from any heretofore investigated.

It is noteworthy that in the fermentation by *Aerobacter*, ethyl alcohol was formed which did not contain fixed carbon. This fact indicates that the alcohol was not from the same source as the acetate containing fixed carbon. These bacteria under proper conditions reduce acids to alcohols (73) but apparently did not do so in the present experiment.

Carbon to Carbon Linkage, Both Components from Carbon Dioxide.—None of the typical heterotrophic bacteria studied by Slade *et al.* (15, 52), formed a carbon to carbon linkage in which both members of the link were from carbon dioxide. It may be a characteristic of most heterotrophs that one member of the link must be organic. In contrast, the typical autotroph uses inorganic carbon for both members of the link. That there are intermediate types is not unexpected. Barker and his co-workers (18) have made an interesting contribution concerning this intermediate group. With radioactive carbon it was shown that the formation of acetic acid by *Clostridium acidii-urici* involves fixation of carbon dioxide. By use of the degradation reactions described above 67 per cent of the radioactive carbon was demonstrated in the methyl and 33 per cent in the carboxyl groups. The unequal distribution of the fixed carbon in the two groups of the molecule may indicate that the acid is not synthesized entirely from carbon dioxide. There is no information available on the mechanism of this fixation. Its solution, obviously, is of fundamental importance.

The organism studied by Wieringa (19) apparently falls in the same cate-

gory as that of Barker *et al.*, only it is even more autotrophic. It reduces carbon dioxide with molecular hydrogen to acetic acid.

Barker and his co-workers (18, 37) claim to have shown fixation of carbon dioxide in cell protoplasm. The mechanism of this fixation has not been investigated, and the type of linkage is unknown.

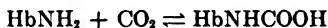
III. Mechanism of Carbon Dioxide Fixation by Animal Tissue

Understanding of the mechanism of fixation of carbon dioxide by bacteria is admittedly inadequate but the situation is even less satisfactory for animal tissue. It has been known since 1935 that even the more fastidious heterotrophs can utilize carbon dioxide. Although the true significance and the fundamental importance of the phenomenon were not generally recognized, there was considerable attention devoted to it by a number of bacteriologists. It is, however, only within the last year that any extensive consideration has been given to the potentialities of carbon dioxide assimilation by animals. True, Krebs and Henseleit (74) in 1932 offered proof of the participation of carbon dioxide in the formation of urea, but the significance of the conversion was largely overlooked. The possibilities of carbon dioxide fixation by animals, therefore, have been investigated to only a very limited extent. Much has been accomplished on the fixation of carbon dioxide by pigeon liver in the oxidation of pyruvate, because the groundwork had been laid by bacterial studies, and the course of action to be followed was apparent. The scope of fixation by animal tissues has by no means been completely probed, and it is very probable that carbon dioxide will be found to have a function in a number of physiological processes.

The same outline will be followed in considering the mechanism of fixation by animal tissue as was used for bacteria, *i. e.*, (1) fixation of carbon dioxide not involving a carbon to carbon linkage; (2) fixation of carbon dioxide involving a carbon to carbon linkage, under which will be considered (a) C_3 and C_4 addition, and (b) miscellaneous fixation reactions.

A. FIXATION OF CARBON DIOXIDE NOT INVOLVING A CARBON TO CARBON LINKAGE

Two examples of such a synthesis are known, formation of carbamino-hemoglobin (75)



and of urea (74). The demonstration by Krebs and Henseleit of urea syn-

thesis (Fig. 3) from carbon dioxide by liver tissue was the first clearly defined example of heterotrophic utilization of carbon dioxide. Conclusive

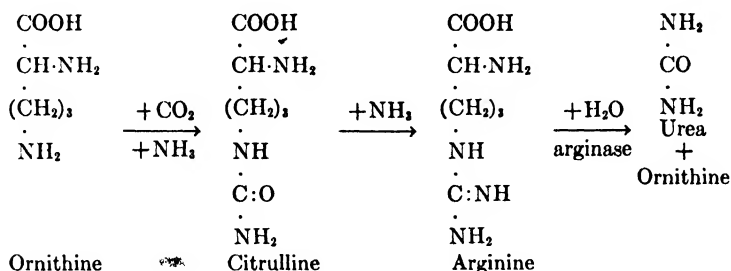


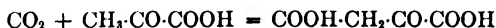
Fig. 3.

proof that carbon dioxide is fixed in urea by liver has been provided by Rittenberg and Waelsh (76) by use of C^{13} and by Evans and Slotin (77) with C^{11} . Hemingway (private communication) has demonstrated urea synthesis *in vivo* with mice by use of C^{13} . In this connection it is interesting to note that the demonstration of Ruben and Kamen (17) of fixation of carbon dioxide by liver has been cited (53, 55) as evidence for mechanisms of carbon dioxide fixation by liver which involve a carbon to carbon linkage. It is evident that the observed fixation could have been due to urea formation. Therefore, the demonstration of fixation, unaccompanied by identification of the compound concerned, does not give reliable evidence for the suggested reactions.

B. FIXATION OF CARBON DIOXIDE INVOLVING A CARBON TO CARBON LINKAGE

1. C_3 and C_1 Addition

The evidence for the occurrence of C_3 and C_1 addition in bacterial metabolism has already been considered and particularly the evidence for the reaction:



The reader is referred to this discussion (p. 153) for a more detailed account concerning the above fixation reaction, for despite Krebs' statement, no direct evidence has been obtained as yet with animal tissue which permits the definite conclusion that pyruvic or oxalacetic acid is a component of the fixation reaction.

Apparently carbon dioxide fixation with formation of a carbon to carbon linkage has been demonstrated in only one tissue—liver. This may be because other tissues have not been examined; it is known, however, that there is no fixation of carbon dioxide during the dissimilation of pyruvate by pigeon breast muscle (63). In liver fixed carbon has been demonstrated in glycogen (53) and in products of pyruvate oxidation (3, 78, 63, 50).

Fixation of Carbon Dioxide in the Dissimilation of Pyruvate by-Liver Tissue.—Attention was focussed on this fixation particularly by the experiments of Evans (79), who found that pyruvate is oxidized by pigeon liver even in the presence of malonate, and is converted to C_4 dicarboxylic acids, α -ketoglutaric acid and carbon dioxide. No theory was advanced by Evans (79) to explain the mechanism but viewed in the light of the experiments of Krebs and Eggleston (80) on pyruvate oxidation by pigeon breast muscle, it was evident that pigeon liver very likely possessed a malonate-insensitive mechanism for formation of C_4 dicarboxylic acids and dissimilated pyruvate by the Krebs cycle. With pigeon breast muscle it was necessary to add a C_4 dicarboxylic acid to the malonate-inhibited reaction in order to get oxidation of pyruvate. Apparently breast muscle cannot synthesize C_4 dicarboxylic acids under the conditions as can liver. The only malonate-insensitive reaction that had been described in the literature for synthesis of C_4 dicarboxylic acids is the fixation reaction studied by Wood and Werkman (41, 42). The fact that oxalacetic acid is an intermediate in the Krebs cycle further added to the attractiveness of the hypothesis that the oxalacetate is formed by the fixation reaction occurring in the dissimilation of pyruvate by pigeon liver. Independently Evans and Slotin (3) and Krebs and Eggleston (55) and shortly afterwards Wood *et al.* (78, 50), presented evidence that carbon dioxide may be fixed by C_3 and C_1 addition in pigeon liver.

Krebs and Eggleston, handicapped by not having an available source of carbon isotopes, were forced to rely on indirect methods of demonstrating the role of carbon dioxide in the pyruvate oxidation. They showed that the rate of oxidation of pyruvate and formation of α -ketoglutarate, citrate, malate and fumarate was stimulated by the presence of carbon dioxide. This specific effect of carbon dioxide in connection with other considerations led them to propose that oxalacetate is synthesized by the Wood and Werkman reaction and then is metabolized by the Krebs cycle. The proposed role of thiamin in this reaction has been considered in connection with results obtained with bacteria (page 158).

Evans and Slotin (3) independently provided conclusive evidence that carbon dioxide is fixed during the oxidation of pyruvate by isolation of

radioactive α -ketoglutarate from experiments in which $C^{14}O_2$ was used as a tracer. Following this Wood *et al.* (78, 50), with heavy carbon, and independently Evans and Slotin (63) with radioactive carbon, determined the position of the carbon fixed in isolated α -ketoglutaric acid. The isolated acid was degraded by acid permanganate oxidation to succinic acid and carbon dioxide.



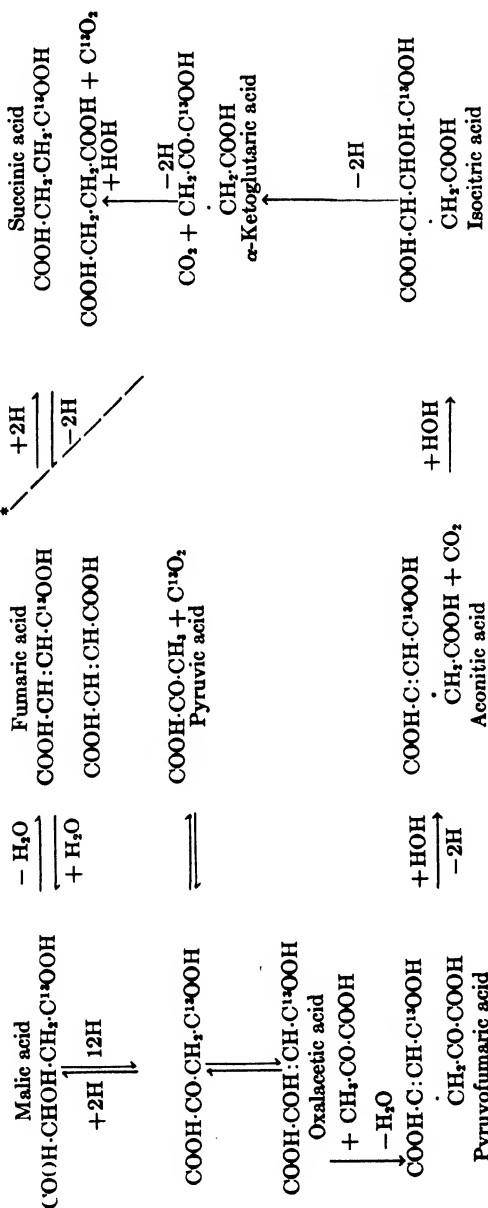
All the fixed carbon was in the carbon dioxide. The identical results of both investigations prove conclusively that the fixed carbon is exclusively in the carboxyl group alpha to the keto group. Krebs (56), convinced that pyruvate was dissimilated in pigeon liver by a combination of oxalacetate synthesis through the fixation reaction and the Krebs cycle, had predicted that the fixed carbon would be found in both carboxyls of the α -ketoglutarate. This, in fact, would be the location of the fixed carbon if pyruvate were fermented by pigeon liver according to the Krebs cycle, since it includes the symmetrical citrate molecule as an intermediate. Wood *et al.* (78), and Evans and Slotin (63), therefore concluded that citrate is not an intermediate in the dissimilation of pyruvate by pigeon liver.

Evans and Slotin (63) have provided further proof that citrate is not an intermediate. Non-radioactive citrate was added to a dissimilation of pyruvate in a radioactive bicarbonate medium and the α -ketoglutarate was isolated. Neither the yield of ketoglutarate nor the ratio of its activity to that of the medium was affected. If citrate were an intermediate in the formation of α -ketoglutarate, it would be expected that the activity of the ketoglutarate would have been lowered due to dilution by ketoglutarate from the citrate.

The status of the Krebs cycle with respect to liver is placed in doubt since citrate is not an intermediate in the dissimilation. Even as applied to pigeon breast muscle the cycle must be accepted with reservations. Unquestionably Krebs' fine investigations have established the general skeleton of the cycle but further investigation is needed to determine the identity of the intermediates. Furthermore, the proof that α -ketoglutarate contains fixed carbon by no means establishes the fixation of carbon dioxide by pigeon liver through C_3 and C_1 addition.

Further investigations by Wood *et al.* (50), on the dissimilation of pyruvate by pigeon liver have more nearly completed this proof, and additional support has been obtained for a modified Krebs cycle.

FIG. 4. DISSIMILATION OF PYRUVATE BY PIGEON LIVER



* Reaction is inhibited by malonate.

Figure 4 presents a tentative mechanism which is adaptable to the observed facts. It is a Krebs cycle from which citrate has been deleted and isocitrate retained. It is recognized that most tissues contain aconitase which would induce formation of citrate, but as a working hypothesis the skeleton of the original cycle may as well be retained until information is available which dictates the proper change. It is probable that phosphorylated intermediate compounds are involved.

Three new facts were established in this study and are related to the scheme as follows:

(a) *Carbon dioxide was found to be fixed exclusively in the carboxyl groups of C₄ dicarboxylic acids formed from pyruvate.* Malate, fumarate and succinate were isolated and degraded. The α - and β -carbons were found free from fixed carbon, which was contained in the carboxyl carbons. This observation more nearly completes the proof that the C₄ dicarboxylic acids are formed by C₃ and C₁ addition, since the fixed carbon was located directly in the carboxyls of the C₄ dicarboxylic acids. The fixation reaction is the only malonate-insensitive reaction that has been proposed which accounts for these facts. Acetic acid condensation, for example, could not account for the observations, even if an exchange of carbon dioxide with the carboxyl carbons were assumed, because this conversion passes through succinate prior to malate and fumarate formation. This apparently was not the case, for it was shown that the conversion of succinate to fumarate can be blocked without inhibition of the formation of malate or fumarate. If there were interconversion of succinate and fumarate, both acids would contain fixed carbon. This was not always the case.

(b) *In the presence of malonate, succinate was shown to contain little or no fixed carbon, whereas the other C₄ dicarboxylic acids did contain fixed carbon.* This observation is of considerable importance. In fact Krebs (81) has referred to the oxidative formation of succinate from oxalacetate in the presence of malonate as the crucial experiment. It is crucial, for, provided one can assume that the anaerobic formation of succinate by reduction of oxalacetate is inhibited by malonate, it proves that there is an oxidative reaction leading from oxalacetate to succinate. Critics of the Krebs cycle (65) have contended that this assumption is without adequate proof. The second fact shows that in the case of pigeon liver, inhibition by malonate was effective and succinate did not arise by anaerobic reduction over malate and fumarate. If such were the case, the C¹³ concentration would have been approximately the same in each compound, as it was when malonate was not added. It is clear that there are two mechanisms for the formation of C₄ dicarboxylic acids. The one is quite probably by C₃ and C₁ addition and is not inhibited by malonate. The other is

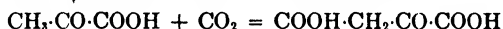
by an oxidative process, and the resulting C_4 dicarboxylic acids do not contain fixed carbon. This then removes one of the major criticisms of the Krebs cycle, at least as applied to the dissimilation by liver. Much of the criticism of the Krebs cycle has centered around this point, and whether or not citrate is an intermediate. In so far as liver is concerned, the first criticism is not valid, while the second is. However, in viewing objections to the cycle, the fact must not be overlooked that this scheme does give a fairly logical mechanism for oxidation of pyruvate to carbon dioxide. The scheme should not be considered a substitute for the Szent-Györgyi system of hydrogen transfer, for the latter scheme only attempts to tell where the hydrogen goes and not how the carbon chain is cleaved. No other scheme adequately explains the mechanism of oxidation of pyruvate to carbon dioxide, and although certain details of the Krebs cycle may be in error, it seems probable that the general framework is correct. At any rate the scheme in Fig. 4 accounts for all observed facts in so far as location of the fixed carbon is concerned. Additional information on the fixation reaction as such has been given in the section dealing with bacteria (page 153).

(c) *Lactate formed either aerobically or anaerobically contained fixed carbon in the carboxyl group and none in the α - and β -carbons.* Further study is required before the significance of these results can be evaluated fully. Probable mechanisms of the formation of lactate containing fixed carbon have already been considered in the section on bacteria (page 166).

A logical objection can be raised to the scheme in Fig. 4 which assigns an essential role to a fixation reaction solely on the basis of the presence and location of fixed carbon in a compound. Fairly reliable information on the mechanism of fixation can be obtained, but the answer as to whether or not the process as a whole is dependent on the fixation reaction cannot be obtained by this method. The carbon may have been fixed, for example, by a non-essential exchange reaction. Some attempt has been made to answer this criticism. For example, the general occurrence of exchange during decarboxylation has been disproved by Krampitz *et al.* (59). Evans and Slotin (63) have considered the problem in some detail. They have presented evidence that the utilization of carbon dioxide is stoichiometric, one molecule of carbon dioxide being utilized for each molecule of α -ketoglutarate. Wood and his co-workers (14) also have shown that there is a stoichiometric relationship of carbon dioxide to propionic and succinic acids formed in the fermentation of glycerol by the propionic acid bacteria. If there is fixation by exchange, it seems questionable whether the reaction would be stoichiometric.

Evans and Slotin (3) have found that α -ketoglutarate formed by breast muscle in the presence of radioactive carbon dioxide is inactive. In muscle, C_4 dicarboxylic acids cannot be formed from pyruvic acid by a fixation reaction; however, if carbon enters the molecule through miscellaneous exchange reactions, there should have been fixation in α -ketoglutarate even in the absence of the true fixation reactions. These results are indicative but admittedly are not conclusive proof that fixation is an essential reaction. There may be a specific fixation by C_3 and C_1 addition and the reaction still be non-essential. For example, the initial oxalacetate synthesis might occur by some other reaction than fixation, and then the carboxyl groups come to equilibrium with the isotopic carbon dioxide by the enzyme exchange reaction of Trampitz *et al.* (59). The C^{13} concentration in the resulting oxalacetate would be the same as if it arose initially by the fixation reaction, and, furthermore, if one blocked fixation the synthesis of oxalacetate and α -ketoglutarate would still go on. There is ample evidence, however, that carbon dioxide does not function solely in a non-essential exchange reaction for it is known to be necessary for growth of microorganisms (22) and in the reduction of methylene blue by dehydrogenases (27).

In summary, the results obtained on fixation of CO_2 by pigeon liver in pyruvate dissimilation, indicate that the CO_2 is fixed by C_3 and C_1 addition, just as in bacterial metabolism. The fact that the fixed carbon of the C_4 dicarboxylic acids has been shown to be exclusively in the carboxyl group supports this view. Proof of fixation of CO_2 in oxalacetate is needed, however, to confirm the occurrence of the reaction:



in liver. Although it is likely that C_4 dicarboxylic acids can be formed by fixation, it is not certain whether this is an essential part of the dissimilation or not.

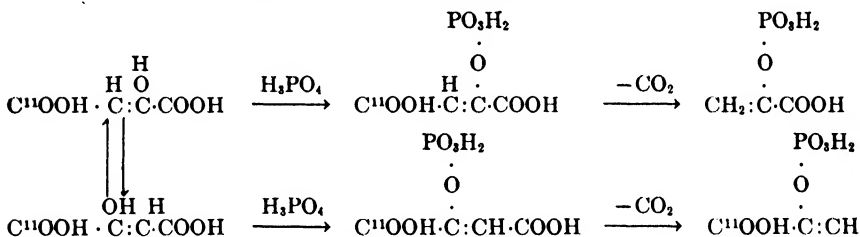
2. Miscellaneous Fixation Reactions

Fixation of Carbon Dioxide in Glycogen by Liver Tissue.—Solomon and his co-workers (53) in an interesting investigation have found that carbon dioxide is fixed *in vivo* by fasted rats when lactate is fed and radioactive sodium bicarbonate is injected intraperitoneally. Part of the fixed carbon occurs in the liver glycogen. The following are the more pertinent results.

The liver glycogen derived from fixed carbon dioxide was calculated to vary from 7 to 16 per cent. If one carbon atom in six of the glycogen had

such an origin, the value would be 16.6 per cent. The calculation was made (a) on the assumption that the $C^{11} : C^{12}$ ratio is the same in tissue fluid in all parts of the body at any particular moment and (b) that the ratio of carbon dioxide excretion to carbon dioxide synthesis into glycogen is constant. If these assumptions hold, then the mM of carbon dioxide incorporated into the glycogen are equal to the mM of excreted carbon dioxide multiplied by the ratio of radioactivity in the glycogen to the radioactivity in the excreted carbon dioxide. The truth of these assumptions is of considerable importance because much of the evidence for the proposed mechanism of fixation is based on the incidence of not more than one carbon atom derived from carbon dioxide in six of the glycogen.

The fixation reaction is proposed by Solomon *et al.*, to constitute an essential step in the glycogen synthesis. The steps of glycolysis are all reversible (82) except that involving phosphopyruvate (83). It is suggested that the phosphopyruvic acid is formed through the fixation reaction by decarboxylation of the phosphorylated C_4 dicarboxylic acid (84, 85).



A random distribution of the C^{11} in the two carboxyl group of the dicarboxylic acid may arise from the shift of hydroxyls in enol oxalacetate (54) or by passage through fumarate. Each pair of phosphopyruvate molecules transformed into glycogen would, therefore, contain one labelled carbon atom. Significance is given to the fact that the experimental value never exceeded this but did approach it rather closely in three of the seven experiments. The above proposal is an attractive explanation of the results but, as Solomon and his co-workers suggest, it should be considered only as a working hypothesis. The fixation reaction, in the first place, has not been elucidated as to details of mechanism; second, the evidence of Kalckar (84) that phosphopyruvate is formed from C_4 dicarboxylic acids is only presumptive since the compound was not isolated; and finally Solomon and his co-workers have indicated a possible inconsistency. They find

that about the same amount of carbon dioxide is fixed in liver glycogen after feeding glucose as after feeding lactate. Accordingly it must be assumed that the glucose is broken down to pyruvate before glycogen is synthesized. This seems unlikely for the path of breakdown of glucose would be through the same phosphorylated hexose as would be needed for the glycogen synthesis. It might be expected that part of the glucose after phosphorylation will go directly to glycogen and that, therefore, the amount of carbon dioxide fixed would be less than when lactate was fed.

Several other observations by Solomon *et al.*, are of interest. Substantially no fixation in glycogen was observed when the rats were not fed lactate, and radioactive sodium bicarbonate was injected into non-fasted rats. Apparently the animal must be actually making and depositing glycogen before there is fixation in the glycogen. The muscle glycogen, in contrast to liver glycogen, contained no fixed carbon. It is apparent, therefore, that there was no significant interchange between liver and muscle glycogen and that the muscle glycogen did not have an origin similar to that of the liver glycogen.

Fixation of Carbon Dioxide by Trypanosoma.—Searle and Reiner (87) found that in the dissimilation of glucose by *Trypanosoma lewisi* carbon dioxide is fixed with formation of succinic, lactic, pyruvic and acetic acids. The carbon dioxide assimilated was equivalent to the succinic acid formed. One molecule of carbon dioxide was assimilated for each two molecules of glucose fermented. Glucose was not metabolized in the absence of bicarbonate unless pyruvate was added. The activating effect of pyruvate differed from that of bicarbonate since succinate was not formed in the absence of bicarbonate and the products were principally lactate with some acetate.

It was suggested that two results were not in agreement with the Wood and Werkman reaction: (a) pyruvate remains unchanged in the presence of bicarbonate, and (b) carbon dioxide is required for the dissimilation of glucose and especially glycerol under aerobic conditions. With glycerol there was no evidence that either pyruvate or succinate was formed as an intermediate or final product; the oxidation goes completely to CO_2 .

Although CO_2 may participate in a manner other than through C_3 and C_1 addition in the oxidation of glycerol, the above evidence is not entirely conclusive. It is only by special methods that intermediate products can be demonstrated. This is the case in the oxidation of pyruvate by liver tissue, yet it is very probable that CO_2 functions by C_3 and C_1 addition. Whether a product will accumulate will depend on whether its rate of formation is more rapid than its rate of oxidation to CO_2 . In the case

of the glycerol oxidation the rates may be such as to cause only CO_2 to accumulate.

Failure to observe dissimilation of pyruvate in the presence of CO_2 may be explained. For example, C_3 and C_1 addition may have occurred but pyruvate may not function as an adequate hydrogen donor to reduce the oxalacetate. The reaction might therefore stop after slight accumulation of oxalacetate. Furthermore, the reaction of pyruvate and CO_2 is merely a picture of the over-all conversion and pyruvate as such may not be an actual component of the fixation reaction.

Probably both the fixation of CO_2 by *Tr. lewisi* and by liver in glycogen, which here are classified as miscellaneous fixation reactions, may with more complete data be shown to fall in the category of C_3 and C_1 addition.

Unidentified Fixation of Carbon Dioxide.—Aside from the specific studies on fixation of carbon dioxide in urea, dicarboxylic acids and glycogen, there have been indications of additional fixations. How many of these are by types already studied is not known. Solomon and his co-workers (53), for example, could not account for 39 per cent of the administered C^{11} and believed that much of it was fixed in organic compounds. Evans and Slotin (63) found that a part of the fixed carbon dioxide that could not be accounted for as α -ketoglutarate was released as carbon dioxide by treatment with ninhydrin and with chloramine T. This fact suggests that there is fixed carbon in amino acids or similar compounds. Such a fixation can readily be explained on the basis of the existence of transaminase in liver and its action on oxalacetic acid and α -ketoglutaric acid (86). There seems little doubt that as our investigations of carbon dioxide fixation are broadened we shall find an ever-widening field of its application.

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ATMUNG, GÄRUNG UND DIE SICH DARAN BETEILIGENDEN ENZYME VON ASPERGILLUS

Von

HIROSHI TAMIYA

Tokyo, Japan

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I. Einleitung

Wenn ich der Aufforderung der Herausgeber, in diesem Handbuch über die enzymatische und physiologische Tätigkeit von *Aspergillus* zu schreiben, Folge leiste, so macht es mir ein Vergnügen, daran zu denken, dass schon seit Jahrtausenden eine besonders intime Verbindung zwischen dem Leben dieses Pilzes und den ostasiatischen Völkern besteht. Bei uns war es seit uralter Zeit—man weiss nicht genau, auf welche Zeit es wirklich zurückgeht—Sitte, den Pilz *Aspergillus oryzae* zum Zweck der Zubereitung und Verarbeitung der Nahrungsmittel und Getränke in Massen zu kultivieren, in der Weise, dass man den gekochten Reis (oder auch manchmal Gerste) mit den Pilzsporen impfte und ihn in einer primitiven Brutkammer stehen liess. Die Massenkultur von *Aspergillus oryzae* auf Reis wird Koji genannt, etymologisch lautet es etwa "Kabi-tachi" oder Pilzanwuchs, zusammengezogen zu "Koji," wovon der japanische Name dieses Pilzes "Koji-Kabi" herrührt. Bei einem gewissen Stadium des Pilzanwuchses wird der Koji einer weiteren Verarbeitung unterworfen, und zwar unter Umständen durch Mischung mit anderen Nahrungsmitteln, wie Sojabohnen. Es tritt dann Gärung durch andere Mikroorganismen ein, und durch weitere Verarbeitungen des Gärgutes werden verschiedene

Getränke (u.a. Sake-Wein), Genuss- und Nahrungsmittel (Shoyu-Sauce, Miso u.a.) hergestellt, die für das Leben der orientalischen Völker nicht nur als Nahrung oder Leckerbissen schlechthin, sondern auch manchmal als Quelle verschiedener Vitamine unentbehrlich sind.

Die Rolle von *Aspergillus oryzae* bei dieser Gärung besteht in erster Linie —analog der Wirkung von Malzamylase bei der Bierbrauerei—in der amylatischen Spaltung von Stärke, wodurch die darauffolgende Gärung durch andere Mikroorganismen ermöglicht wird. Nähere analytische Untersuchungen haben aber gezeigt, dass zum Reifungsprozess des Gärgutes nicht nur die diastatische Wirkung allein, sondern auch vielfach anderweitige enzymatische sowie physiologische Tätigkeiten von *Aspergillus* beitragen.

Schon früher hat man auf die ausgesprochene Vielseitigkeit der hydrolatischen Wirksamkeit von *Aspergillus* aufmerksam gemacht, und besonders seit der Darstellung des Enzympräparates Takadiastase* von J. Takamine (87, 88) im Jahre 1894 ist dieser Pilz wegen seines ungeheueren Reichtums an hydrolytischen Enzymen ein allgemeines Versuchsmaterial der Enzymologen geworden. Bis jetzt sind darin mehr als 50 verschiedene Arten der Enzyme nachgewiesen, und es wäre freilich keine Übertreibung zu sagen, dass es überhaupt keinen Organismus gibt, dessen Enzyme so vielseitig untersucht worden sind und in welchem so viele Arten von Enzymen aufgefunden worden sind wie in *Aspergillus oryzae*.

Merkwürdig ist aber nun der Umstand, dass die enzymologischen Studien von *Aspergillus* bisher fast ausschliesslich auf dessen hydrolytische Enzyme beschränkt sind, während seine desmolytischen sowie oxydoreduktiven Enzyme, die in den physiologischen Leistungen dieses Pilzes eine grundlegende Rolle spielen, nur wenig Berücksichtigung gefunden haben. Ein besonderer Aspekt, der ein lebhaftes Interesse der Biochemiker auf *Aspergillus* gelenkt hat, ist sein Vermögen zur sogen. "oxydativen Gärung," d.i. die Fähigkeit, in seinem Stoffwechsel verschiedene organische Säuren zu bilden. In der Literatur findet man eine beträchtliche Zahl von Beschreibungen darüber, ob und unter welchen Bedingungen verschiedene Säuren wie Glucon-, Citronen-, Koji- und Oxalsäure in wechselnden Mengen angehäuft werden. Trotz zahlreichen experimentellen Untersuchungen und wiederholten Auseinandersetzungen unter verschiedenen Forschern bleibt aber heute die physiologische Bedeutung sowie der chemische Mechanismus der Säurebildungsvorgänge von *Aspergillus* noch vielfach unaufgeklärt, was meines Erachtens vor allem darauf beruht, dass die Probleme meist allzu sehr deskriptiv-biochemisch, und nicht gebührend physiologisch-enzymologisch unter Berücksichtigung der Wirkung von desmolytischen sowie oxydoreduktiven Enzymen erforscht worden sind.

Im Folgenden ist beabsichtigt, von den speziellen Fragen der Säurebildung und dergleichen spezifischen physiologischen Erscheinungen Abstand nehmend, in der Hauptsache die allgemeingültigen physiologischen Leistungen des Pilzes wie Atmung, alkoholische Gärung und Wachstum von

* Der chinesischen Literatur zufolge hat man dort schon seit geraumer Zeit den Koji, analog wie die heutige Takadiastase, als Heilmittel gegen Magenkrankheiten benutzt, und zwar mit der ehrerbietigen Bezeichnung: "Gottes-Koji."

möglichst umfassenden Gesichtspunkten aus unter Berücksichtigung der sich daran beteiligenden Enzyme zu betrachten. Als Gegenstand sollen hauptsächlich diejenigen Arten von *Aspergillus* in Betracht kommen, die, wie die meisten anderen Organismen, die zu Gebote stehende C-Quelle praktisch vollständig zu Kohlensäure und Wasser veratmen, also die *Aspergilli*, die keine oder nur schwache Säurebildner sind. Der Darstellung liegt nämlich der Gedanke zugrunde, dass die mannigfaltigen Säurebildungserscheinungen, deren Art und Weise nicht nur je nach den Pilzarten und -rassen, sondern auch je nach den Kulturbedingungen weitgehend veränderlich sind, wohl irgendwelche modifizierte Teilerscheinungen des Atmungs- oder Wachstumsstoffwechsels des Pilzes darstellen, dass also allerlei Einzelprobleme der Säurebildung u.a. stets im Zusammenhang mit solchen grundlegenden physiologischen Leistungen des Pilzes erfasst und gedeutet werden müssen.

Die erste Hälfte der Darlegungen soll der allgemeinen physiologischen Betrachtung des Atmungs- und Wachstumsstoffwechsels gewidmet werden, von dem Gesichtspunkte ausgehend, dass auch unser Versuch, die in Betracht kommenden physiologischen Grundleistungen in einzelne Teilvorgänge zu zerlegen, und womöglich den Mechanismus der einzelnen Teilprozesse enzymologisch zu ergründen, wohl erst aus der Erkenntnis der physiologischen Grundlage fruchtbar erzielt werden kann. Mit der Frage der Atmungsenzyme und der Dehydrasen des Pilzes wird sich der zweite Teil der Abhandlung beschäftigen.

II. *Aspergilli* als strenge Aerobionten und ausgeprägte Omnivoren

Aspergillus, wie alle Schimmelpilze überhaupt, ist ein streng aerobes Lebewesen, das sich nur in Anwesenheit von Sauerstoff fortpflanzen kann. Ausgesprochene Aerophilie von diesem Pilz äussert sich darin, dass er normalerweise nur auf der Oberfläche der Nährböden (seien sie flüssig oder fest) unter Bildung der sogen. Pilzdecke wächst. Diese Decke besteht gewöhnlich aus mehreren heterogenen Schichten; die unterste Schicht, welche eine dicht verknäuelte Hyphenmasse darstellt, ist in die Nährflüssigkeit eingetaucht, während die oberste, in dem Luftraum befindliche Schicht aus locker verfilzten Mycelien (Lufthyphen) besteht, wovon je nach den Bedingungen Konidiosporenträger aussprossen. Die Atmung sowie das Wachstum wird hauptsächlich von diesen Lufthyphen vollzogen.

Unter besonderen Verhältnissen kann der Pilz auch submers innerhalb der Nährflüssigkeit wachsen, wobei die Mycelien sich nicht dicht miteinander verwickeln, sondern locker verflochtene, kleine Hyphenfitz-

chen bilden. Die Aufschlammung der submersen Hyphen, die in verschiedener Hinsicht abweichendes physiologisches Verhalten von demjenigen des Deckenpilzes aufweisen, erhält man im Laboratorium entweder durch dauerndes Schütteln der Kulturflüssigkeit während der Züchtung (Kluyver und Perquin (34)) oder durch ständiges Umrühren der Kulturlösung unter Durchleitung von Luft (Ogura und Nagahisa (71)).

Ein besonderes Charakteristikum der Schimmelpilzatmung ist ihre auffällende Empfindlichkeit gegenüber der Schwankung des O_2 -Drucks, worauf schon 1904 von T. Porodko (74) aufmerksam gemacht wurde. Bei der Deckenkultur von *Aspergillus oryzae* wurde festgestellt, dass die maximale Atmung (Rohrzucker als Substrat) erst bei Zufuhr von etwa 67–83% (d.i. ca. 500 bis 630 mm. Hg) Sauerstoff erreicht wird, während bei Zufuhr von 30–50% (230 bis 380 mm. Hg) oder weniger O_2 , fast proportional der Erniedrigung des Sauerstoffdrucks immer kleinere Atmungsgrösse beobachtet wird (Tamiya (95)). Gegenüber dem Deckenpilz erweisen sich merkwürdigerweise die durch Durchlüftungskultur erhaltenen submersen Hyphen weniger empfindlich gegen O_2 -Druckerniedrigung, indem erst bei 10% O_2 eine bemerkbare Herabsetzung der Atmung eintritt (Ogura und Nagahisa (71)). Selbst dieser Wert ist doch bei weitem höher als der bei anderen submers vegetierenden Mikroorganismen beobachtete kritische O_2 -Druck der Atmung, der z.B. bei *Microc. candidus* unter gewissen Bedingungen niedriger als 10^{-6} Atm. (0.008 mm. Hg) ausfallen kann (Warburg und Kubowitz (124)).

Seit langem ist die Tatsache bekannt gewesen, dass *Aspergillus* sehr viele Arten der Kohlenstoffverbindungen als C-Quelle zu verwerten vermag. Systematische Untersuchungen in dieser Richtung wurden u.a. von H. Tamiya (98); Ogura und Nagahisa (71); Tamiya und Usami (107) an *Aspergillus oryzae* ausgeführt,* und zwar unter besonderer Berücksichtigung der verschiedenen Verwertbarkeit der einzelnen C-Quellen im Atmungs- und Wachstumsstoffwechsel des Pilzes. Zur Erprobung gelangten insgesamt etwa 140 Arten von Substanzen, von welchen 75 mehr oder weniger gut verwertet wurden. Als gute C-Quellen für Wachstum und Atmung erwiesen sich u.a. folgende Substanzen:†

* Über die Versuchstechnik, unter anderem über den Respirometer, der von uns besonders zum Zweck der Pilzstoffwechsel-Untersuchung konstruiert wurde, sei auf die Beschreibungen von Tamiya (95, 98) verwiesen. Alle Versuche von Tamiya und seinen Mitarbeitern, die hier und im nachfolgenden angeführt werden, wurden unter Anwendung dieses Respirometers ausgeführt.

† Der Q_{O_2} -Wert betrug bei solchen C-Quellen ca. 16–45. Es gibt noch eine Anzahl von Substanzen, die von anderen Autoren als verwertbare C-Quellen für *Aspergillus* bestätigt worden sind; vergleiche hierzu die zusammenfassende Darstellung von Tamiya (98). Wie ausgiebig die Polyphagie dieses Pilzes entwickelt ist, ersieht man z.B. aus dem von Tausson (110, 111) sowie von Hopkins und Chibnall (29) erhobenen Befund, dass gewisse *Aspergillus*-Arten auf höheren Paraffinen (mit C-Zahl bis zu 34) sowie auf Bienenwachs gedeihen können.

Kohlehydrate (verschiedene Polysaccharide, Hexosen, Pentosen und Triosen), mehrwertige Alkohole (Glycerin, Erythrit, Adonit, Mannit, Sorbit, Dulcit, Styracit, Quercit, Inosit u.a.), Äthylalkohol, Bernsteinsäure, Azelainsäure, Sebacinsäure, Aconitsäure, Brenztraubensäure, Milchsäure, Äpfelsäure, Citronensäure, Weinsäure, Gluconsäure, Zuckersäure, Schleimsäure, Chinasäure, Salicylsäure, *m*-Oxybenzoesäure, Protocatechusäure, Gallussäure, Kojisäure, Glykokoll, *l*-Alanin, *l*-Serin, *l*-Valin, *l*-Leucin, *l*-Isoleucin, *l*-Asparaginsäure, *l*-Glutaminsäure, *l*-Phenylalanin, *l*-Prolin, *l*-Histidin, *l*-Tryptophan, *l*-Arginin.

Ferner zeigen sich folgende Substanzen als Atmungssubstrat verwertbar, während sie kein oder nur spärliches Pilzwachstum gestatten.

Äthylenglykol, Trimethylenglykol, Essigsäure, Glykolsäure, Ameisensäure, β -Oxybuttersäure, Hydrochinon, β -Resorcylsäure.

Gegenüber verschiedenen Substanzen verhalten sich die durch Durchlüftungskultur erhaltenen submersen Hyphen im grossen und ganzen gleich wie die Myceldecken. Ausnahme machen dabei die Befunde bei Lävulinsäure, Pentaerythrit, Glutarsäure und Adipinsäure, die vom Deckenpilz fast gar nicht, von submersen Hyphen aber mehr oder minder veratmet werden. Andererseits wurde die Schleimsäure, die durch Deckenpilz gut oxydiert wurde, durch submerse Hyphen gar nicht angegriffen.

Nicht nur gegenüber den C-Quellen, sondern auch gegenüber N-Quellen macht sich der ausgesprochene Omnivorencharakter dieses Pilzes geltend. Es sei hier nur darauf hingewiesen, dass nach F. Czapek (16, 17, 18) aus 196 geprüften N-Verbindungen 127 Substanzen von verschiedenen chemischen Kategorien (Ammon-, Nitrat-, Nitrit-, Hydroxylamin- und Hydrazinsalze, Amide, Aminosäuren, Nitrile, Purin- und Pyrimidinbasen, Azoverbindungen u.s.w.) als N-Quelle von *Aspergillus niger* verwertet werden könne.* Mehrere organische N-Verbindungen (Hippursäure, Harnsäure, verschiedene Aminosäuren u.a.) können gleichzeitig als C- und N-Quellen verwertet werden, sodass durch alleinige Zugabe solcher Substanzen—allerdings in Gegenwart kleiner Mengen der notwendigen Mineralsubstanzen—der Aufbau der ganzen Zellsubstanzen möglich ist.† Für das Wachstum von *Aspergillus* ist die Anwesenheit der bios- oder wuchsstoffartigen Substanzen manchmal günstig, aber in der Regel ganz entbehrlich, was bei seinem langjährigen domestizierten Leben recht merkwürdig ist.

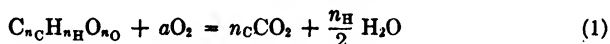
* Vergl. auch Puriewitsch (75), Kossowicz (37, 38, 39, 40) und Raciborski (77).

† Vergl. Kossowicz (39, 40), Nikitinsky (68), Emmerling (22), Tamiya und Usami (107).

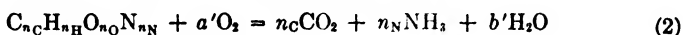
III. Bilanz des Stoffwechsels

Bei den *Aspergillen* vom Nichtsäurebildner-Typ werden die C-Atome in den verbrauchten C-Quellen zum Teil durch Atmung zu CO_2 verwandelt, zum Teil aber auch in dem durch Wachstum neugebildeten Pilzkörper aufgestapelt. Bei Veratmung der N-haltigen C-Quellen wird das N-Atom in den meisten Fällen sämtlich in Form von NH_3 abgespalten (Tamiya und Usami (107)). Die allgemeinen Bilanzgleichungen der Atmung lauten nämlich:

bei den nicht N-haltigen C-Quellen ($\text{C}_{n_C}\text{H}_{n_H}\text{O}_{n_O}$):



bei den N-haltigen C-Quellen ($\text{C}_{n_C}\text{H}_{n_H}\text{O}_{n_O}\text{N}_{n_N}$):



Das Verhältnis $\frac{(\text{abgegebenes CO}_2)}{(\text{aufgenommener O}_2)}$ bei der vollkommenen Verbrennung lässt sich für jede C-Quelle vorausberechnen, wenn uns ihre elementare Zusammensetzung bekannt ist. Diesen theoretischen (CO_2/O_2) -Wert nennen wir den Verbrennungsquotienten und bezeichnen ihn mit CQ ; es gilt dabei folgende Beziehung (Tamiya (99, 102)):

$$CQ = \frac{4n_C}{4n_C + n_H - 3n_N - 2n_O}$$

An *Aspergillus oryzae* wurde unter Zugabe verschiedener Arten von C-Quellen der sogen. respiratorische Quotient, d.i. der physiologische (CO_2/O_2) -Wert, ermittelt, und dies mit dem theoretischen CQ -Wert verglichen (Yamagata (129); Tamiya und Usami (107)). Merkwürdigerweise fielen diese beiden Werte fast nie gleich aus, wie aus der Tabelle I hervorgeht.

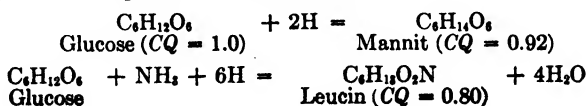
In dieser Tabelle bedeutet RQ den respiratorischen Quotienten, der bei Zugabe von NH_4Cl als N-Quelle bzw. bei Zugabe der Aminosäuren als alleinige C- und N-Quelle beobachtet wurde, während RQ_{NO_3} denjenigen Wert darstellt, der sich bei Zugabe von KNO_3 als N-Quelle ergab. RQ_{NO_3} fällt stets um einen gewissen Betrag grösser als RQ aus. Beschränken wir uns zunächst auf die Betrachtung von RQ , so werden wir einer interessanten Tatsache gewahr, nämlich: bei denjenigen Substanzen, deren CQ -Wert grösser als 0.96 ist, ist fast ausnahmslos $RQ > CQ$, während umgekehrt bei den Substanzen, deren CQ kleiner als 0.92 ist, fast immer $RQ < CQ$ ist; nur bei den Substanzen mit dem CQ -Wert von 0.92–0.96 fällt der RQ -Wert praktisch dem CQ -Wert gleich aus. Diese auffallend regelmässige Erscheinung wurde dahin gedeutet, dass der Wachstumsvorgang des Pilzes, der

TABELLE I
RESPIRATORISCHE QUOTIENTEN BEI ZUGABE VERSCHIEDENER C-QUELLEN

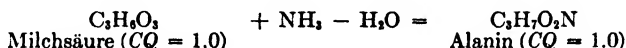
C-Quelle	CQ	RQ	RQ _{NO₂}
Serin	1.20	1.28	...
Histidin	1.20	1.32	...
Gluconsäure	1.09	1.32	1.39
Stärke	1.00	1.11	1.28
Saccharose	1.00	1.22	1.46
Mannose	1.00	1.25	1.57
Glucose	1.00	1.16	1.30
Galactose	1.00	1.15	1.33
Arabinose	1.00	1.12	1.31
Dioxyaceton	1.00	1.09	1.19
Alanin	1.00	0.94	...
Tryptophan	0.96	0.95	...
Mannit	0.92	0.92	1.15
Sorbit	0.92	0.93	1.17
Adonit	0.91	0.86	1.14
Prolin	0.91	0.92	...
Erythrit	0.89	0.85	1.08
Glycerin	0.86	0.77	0.94
Valin	0.83	0.80	...
Leucin	0.80	0.70	...
Isoleucin	0.80	0.71	...
Äthylalkohol	0.67	0.59	0.66

bei allen obigen Versuchen stets mehr oder weniger stark einsetzte, je nach den CQ-Werten der dargereichten C-Quellen entweder eine übermässige O₂-Aufnahme oder aber eine überschüssige CO₂-Abspaltung aus den Substraten mit sich führt, und zwar ist dieser umgekehrte Ausfall gerade davon abhängig, ob der Wachstumsvorgang, d.i. der Aufbau des Pilzkörpers aus seinen Bausteinen, als ganzes einen Oxydations- oder einen Reduktionsvorgang darstellt (Tamiya (99, 102)).

Um das Gesagte sachlicher darzulegen, sei zunächst die Bedeutung des von uns definierten CQ-Wertes betrachtet. Man kann ganz allgemein sagen, dass die Umwandlung der Substanzen mit kleineren CQ-Werten in diejenigen mit grösseren CQ-Werten einen Oxydationsvorgang, die Umwandlung in umgekehrter Richtung einen Reduktionsvorgang darstellt, wie aus folgenden Beispielen ersichtlich ist.



Andererseits kommt bei den Veränderungen, in welchen als ganzes nur Ein- oder Austritt von H_2O bzw. NH_3 in die Moleküle stattfindet, keine Veränderung des CQ -Wertes zustande; so z.B.



Aus dieser Überlegung folgt nun: der Aufbauprozess des Pilzkörpers aus dessen Bausteinen stellt als Ganzes entweder einen Reduktionsvorgang oder einen Oxydationsvorgang dar, je nachdem ob der CQ -Wert der Baustein-C-Quelle grösser oder kleiner als derjenige des Pilzkörpers ist. Es lag also nahe, den CQ -Wert des Pilzkörpers festzustellen, d.i. die elementare Zusammensetzung der Pilzmycelien kennen zu lernen.

TABELLE II
ELEMENTARE ZUSAMMENSETZUNG VON *Aspergillus oryzae*

C-Quelle	N-Quelle	In % der aschefreien Trockensubstanz				"Bruttoformel"	Verbrennungsquotient
		C	H	O	N		
Glucose	NH_4Cl	49.07	7.17	37.29	6.47	$\text{C}_{409}\text{H}_{717}\text{O}_{233}\text{N}_{46}$	0.935
	KNO_3	49.78	7.28	37.99	4.95	$\text{C}_{418}\text{H}_{718}\text{O}_{237}\text{N}_{35}$	0.918
Mannit	NH_4Cl	48.95	7.09	37.04	6.92	$\text{C}_{408}\text{H}_{709}\text{O}_{232}\text{N}_{49}$	0.943
	KNO_3	49.00	7.27	37.69	6.04	$\text{C}_{409}\text{H}_{727}\text{O}_{238}\text{N}_{43}$	0.927
Glycerin	NH_4Cl	49.54	7.00	34.95	8.51	$\text{C}_{413}\text{H}_{703}\text{O}_{218}\text{N}_{61}$	0.953
	KNO_3	49.82	7.48	36.97	5.73	$\text{C}_{418}\text{H}_{748}\text{O}_{231}\text{N}_{41}$	0.911
Äthylalkohol	NH_4Cl	49.37	7.41	35.11	8.11	$\text{C}_{411}\text{H}_{741}\text{O}_{219}\text{N}_{58}$	0.927
	KNO_3	49.45	7.52	36.35	6.68	$\text{C}_{412}\text{H}_{752}\text{O}_{227}\text{N}_{58}$	0.915
Leucin	47.40	7.29	37.22	8.09	$\text{C}_{398}\text{H}_{723}\text{O}_{233}\text{N}_{58}$	0.950

Wie aus Tabelle II hervorgeht, zeigte der Pilzkörper unabhängig von den Kulturbedingungen eine recht konstante Zusammensetzung (Yamagata (128); Tamiya und Usami (107)). Der Verbrennungsquotient des Pilzkörpers berechnet sich daraus zu 0.91–0.95, also ein Wert, der in guter Annäherung dem CQ -Wert von denjenigen C-Quellen entspricht, bei welchen der RQ -Wert praktisch dem CQ -Wert gleichkommt. Die Tatsache, dass der RQ -Wert bei den C-Quellen, deren Verbrennungsquotient grösser als derjenige des Pilzkörpers ist (Hyperquotient), grösser als CQ ausfällt, erklärt sich dadurch, dass der Aufbauvorgang des Pilzkörpers, der hierbei *in toto* eine Reduktion ist, eine Decarboxylierung der C-Quelle nach sich zieht. Bezeichnet man die Bruttoformel der nicht N-haltigen C-Quelle mit $\text{C}_{\text{nC}}\text{H}_{\text{nH}}\text{O}_{\text{nO}}$ und diejenige des Pilzkörpers mit $\text{C}_{\text{rC}}\text{H}_{\text{rH}}\text{O}_{\text{rO}}\text{N}_{\text{rN}}$, so dürfte

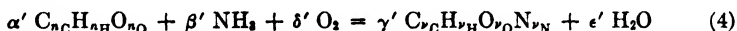
man für den Wachstumsvorgang (NH_3 als N-Quelle) folgende allgemeine Bilanzformel annehmen:*



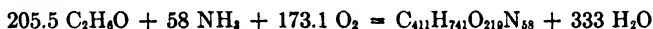
So. z.B. bei Zucker, dessen CQ 1.00 ist:



Der kleinere Ausfall des RQ -Wertes bei denjenigen C-Quellen, deren Verbrennungsquotient kleiner als derjenige des Pilzkörpers ist (Hypoquotient), ist dagegen dadurch zu erklären, dass der Aufbauvorgang, der hierbei als ganzes eine Oxydation darstellt, etwa wie folgt unter O_2 -Aufnahme vollzogen wird:



Beispielsweise lautet die Formel bei Alkohol, dessen CQ -Wert 0.667 ist, folgendermassen:



Ähnliche Bilanzgleichungen können deduktiv für jede andere C-Quelle aufgestellt werden, und aus diesen lassen sich die Mengen des durch Wachstum überschüssig aufgenommenen O_2 bzw. überschüssig ausgeschiedenen CO_2 berechnen.† Wenn je nach der Grösse des CQ -Wertes der C-Quelle

* Der Aschengehalt des Pilzkörpers beträgt höchstens ca. 10% vom gesamten Trockengewicht. Zieht man z.B. nach den Analysendaten von R. Takata (89, 90) den Gehalt an verschiedenen Mineralsubstanzen in Betracht, so lautet die "Bruttoformel" des *Aspergillus*-Körpers wie folgt:



Bei grosszügiger Betrachtung der ganzen Stoffwechselbilanz darf man also die Anwesenheit der Mineralsubstanzen vernachlässigen.

† Bezeichnet man die Mengen (in cc.) des bei Bildung von 1 gm. Pilzkörper überschüssig aufgenommenen Sauerstoffs (bei den C-Quellen mit Hypoquotienten) und überschüssig ausgeschiedenen CO_2 (bei den C-Quellen mit Hyperquotienten) mit λ_{O_2} bzw. λ_{CO_2} , so bestehen folgende Beziehungen:

$$\lambda_{\text{O}_2} = \frac{22410 \cdot r_C}{\Omega \Phi} \left[\frac{\Phi - CQ}{CQ} \right],$$

$$\lambda_{\text{CO}_2} = \frac{22410 \cdot r_C}{\Omega \Phi} [CQ - \Phi],$$

worin CQ = den Verbrennungsquotienten der C-Quelle,

Φ = den Verbrennungsquotienten des Pilzkörpers,

Ω = das "Molekulargewicht" von $\text{C}_{r_C} \text{H}_{r_H} \text{O}_{r_O} \text{N}_{r_N}$,

r_C = die Zahl des in Ω gm. Pilzkörper enthaltenen C-Atoms bedeutet.

entweder die überschüssige CO₂-Abgabe oder die überschüssige O₂-Aufnahme durch den Wachstumsvorgang herbeigeführt werden sollte, so muss die Atmung bei den C-Quellen mit Hyperquotienten nach der Grösse der O₂-Aufnahme, bei den C-Quellen mit Hypoquotienten dagegen nach der Grösse der CO₂-Abgabe ermittelt werden. Aus der experimentell gefundenen Wachstumsgrösse und der Atmungsgrösse können wir unter Berücksichtigung der obigen Wachstumsformeln den RQ-Wert in einzelnen Fällen ausrechnen.* Wie in Tabelle III gezeigt wird, stimmen die berechneten

TABELLE III
RQ-WERTE ALS FUNKTION DER ATMUNGS- UND WACHSTUMSGRÖSSE

C-Quelle	λCO_2	λO_2	λNH_3	Atmungs- grösse (O ₂ - Aufnahme bzw. CO ₂ - Abgabe in cc.)	Wachstums- grösse (Pilz- gewichts- zunahme in mg.)	RQ		NH ₃ -Bildung (in mg.)	
						gef.	ber.	gef.	ber.
Serin	233	...	0.184	16.5	5.9	1.28	1.29
Histidin	233	...	0.326	21.6	9.0	1.32	1.30	10.08	11.57
Glucensäure	117	34.4	60.3	1.32	1.38
Stärke	63.8	22.6	32.0	1.11	1.09
Saccharose	63.8	27.5	38.0	1.22	1.09
Mannose	63.8	35.2	58.5	1.25	1.11
Glucose	63.8	38.1	44.0	1.16	1.08
Galactose	63.8	24.9	41.3	1.15	1.11
Arabinose	63.8	35.8	51.0	1.12	1.09
Dioxyaceton	63.8	12.2	8.0	1.09	1.05
Alanin	46.5	...	0.137	59.0	22.0	0.94	1.02	14.0	17.95
Tryptophan	6.5	...	0.025	28.9	11.8	0.95	0.96	3.73	4.11
Mannit	...	21.0	...	17.9	39.0	0.92	0.89
Sorbit	...	21.0	...	37.6	63.0	0.93	0.89
Adonit	...	36.3	...	25.5	49.8	0.89	0.86
Prolin	...	42.0	0.036	25.0	9.0	0.92	0.90	3.63	4.38
Erythrit	...	58.9	...	26.6	53.8	0.85	0.81
Glycerin	...	109	...	19.1	31.5	0.77	0.74
Valin	...	131	0.036	13.8	8.1	0.80	0.78
Leucin	...	175	0.014	25.4	23.0	0.70	0.71	3.18	3.48
Isoleucin	...	175	0.014	30.7	28.3	0.71	0.71	4.69	4.28
Äthylalkohol	...	388	...	18.5	18.0	0.59	0.53

* Bezeichnet man mit

I_{O_2} = die Menge des insgesamt aufgenommenen Sauerstoffs in cc.,

I_{CO_2} = die Menge der insgesamt ausgeschiedenen Kohlensäure in cc.,

M_0, M = das Pilzgewicht (in gm.) am Anfang bzw. am Ende des Versuchs,

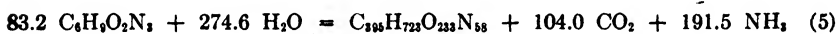
so ist bei den C-Quellen mit Hyperquotienten:

$$RQ = CQ + \frac{(M - M_0)\lambda\text{CO}_2}{I_{\text{O}_2}},$$

(Fortsetzung der Fussnote auf S. 193)

RQ-Werte bei fast allen C-Quellen vorzüglich mit den wirklich gefundenen Werten überein, woraus wir sehen, dass die oben angeführten Formeln die bilanzmässigen Verhältnisse des Wachstumsvorgangs wohl ganz allgemein für verschiedene C-Quellen richtig wiedergeben.*

Bei den Aminosäuren, die beim Pilz gleichzeitig als C- und N-Quelle dienen, verhält sich die Sache im Grunde analog wie oben, nur dass dabei eine besondere Erscheinung hinsichtlich des Umsatzes von N hinzukommt (Tamiya und Usami (107)). Während das N-Atom in solchen Verbindungen, wie schon gesagt, durch Veratmung praktisch gänzlich in Form von NH_3 abgespalten wird, findet auch durch den Wachstumsvorgang eine mehr oder weniger deutliche NH_3 -Abspaltung statt, was daraus ersichtlich ist, dass der relative N-Gehalt bei den meisten Aminosäuren (ausgenommen aber z.B. Phenylalanin) grösser als derjenige vom Pilzkörper ist. Ebenso wie bei den nicht N-haltigen C-Quellen erfolgt der Aufbauprozess aus den Aminosäuren mit Hyperquotienten unter CO_2 -Abspaltung, so z.B. bei Histidin, dessen *CQ*-Wert 1.20 ist:



Dagegen soll bei dem Aufbau aus den Aminosäuren mit Hypoquotienten, wie z.B. Leucin (*CQ* = 0.80), eine O_2 -Aufnahme stattfinden, nämlich:



Die nach solchen deduktiven Formeln berechneten *RQ*-Werte sowie die

(Fortsetzung der Fussnote von S. 192)

bei den C-Quellen mit Hypoquotienten:

$$RQ = \frac{I_{\text{CO}_2}}{(M - M_0)\lambda_{\text{O}_2} + \frac{I_{\text{CO}_2}}{CQ}}$$

* Bei verschiedenen Kohlehydraten fiel der gefundene *RQ*-Wert ein wenig grösser als der berechnete aus, was, wie später ausführlich erwähnt wird, auf das Einsetzen der "aeroben Gärung" zurückzuführen ist. Bei einigen C-Quellen fand neben der eigentlichen Atmung in geringem Masse Kojisäurebildung statt, die auf den *RQ*-Wert verkleinernd wirken sollte. Bei der verwendeten Versuchsanordnung und -dauer war die Menge der Kojisäurebildung so geringfügig, dass sie bei grosszügiger Bilanzanalyse praktisch ausser acht gelassen werden kann (vergl. Tamiya (99)).

NH_3 -Menge* stimmen auch im Grossen und Ganzen mit den experimentell ermittelten Zahlen überein, wie aus Tabelle III ersichtlich ist.†

Gehen wir nun auf die Fälle der Zugabe von Nitrat als N-Quelle über, bei welchen der RQ -Wert stets grösser ausfällt als bei Zugabe von NH_3 . Wie leicht einzusehen, beruht dies darauf, dass die Reduktion des Nitrates zu NH_3 , die bei der Stickstoffassimilation notwendig stattfinden muss, eine überschüssige CO_2 -Abspaltung aus organischen Molekülen verursacht, eine Tatsache, die zuerst von O. Warburg und E. Negelein (126) bei *Chlorella* und später von W. Ruhland und H. Ullrich (79) bei höheren Pflanzen festgestellt wurde. Bei Darreichung des Nitrates zur Pilzkultur, wobei ein ebenso starkes Wachstum wie bei NH_3 -Zugabe stattfindet, wird in der Kulturlösung praktisch keine Spur von NH_3 sowie von irgendwelchen intermediären Reduktionsprodukten von Nitrat nachgewiesen, d.h. das aus Nitrat reduktiv gebildete NH_3 wird sofort restlos assimiliert. Zunächst soll die Frage dahingestellt sein, was für eine Substanz in der intermediären Stufe des Stoffwechsels primär mit der Nitratreduktion gekoppelt dehydriert wird; da in der totalen Stoffwechselbilanz nur die C-Quelle als der endgültige Reduktant gegenüber Nitrat in Betracht kommen kann, und weil als deren Oxydationsprodukt letzten Endes nur CO_2 in Frage kommt,

* Bezeichnet man mit

I_{NH_3} = die Menge (in gm.) des durch die Atmung abgespaltenen Ammoniaks,

λ_{NH_3} = die Menge (in gm.) des bei Bildung von 1-gm. Pilz aus Baustein-Aminosäure abgespaltenen Ammoniaks,

$I\mathcal{Z}_{\text{NH}_3}$ = die Gesamtmenge (in gm.) des gebildeten Ammoniaks,

so ist: bei den Aminosäuren mit Hyperquotienten:

$$I_{\text{NH}_3} = I_{\text{O}_2} \cdot \frac{n_{\text{N}}}{n_{\text{C}}} [\text{CQ}] \times 0.76 \cdot 10^{-3}; \quad \lambda_{\text{NH}_3} = \frac{\nu_{\text{C}}}{\Omega \Phi} \left[\text{CQ} \frac{n_{\text{N}}}{n_{\text{C}}} - \Phi \frac{\nu_{\text{N}}}{\nu_{\text{C}}} \right] \times 17.03,$$

bei den Aminosäuren mit Hypoquotienten:

$$I_{\text{NH}_3} = I_{\text{CO}_2} \frac{n_{\text{N}}}{n_{\text{C}}} \times 0.76 \cdot 10^{-3}, \quad \lambda_{\text{NH}_3} = \frac{\nu_{\text{C}}}{\Omega} \left[\frac{n_{\text{N}}}{n_{\text{C}}} - \frac{\nu_{\text{N}}}{\nu_{\text{C}}} \right] \times 17.03$$

Die Gesamtmenge des gebildeten NH_3 ist dabei:

$$I\mathcal{Z}_{\text{NH}_3} = I_{\text{NH}_3} + (M - M_0)\lambda_{\text{NH}_3}$$

† Nur bei Arginin wurde ausnahmsweise eine merkliche Abweichung zwischen der berechneten und der gefundenen NH_3 -Menge festgestellt. Durch nähere Analyse der Stoffwechselprodukte konnten wir dartun, dass dabei ein Teil der N-Atome im Argininmolekül, und zwar derjenige in der Guanidylgruppe, durch Atmung sowie Wachstum als Harnstoff abgespalten wird, der erst später teilweise durch Pilzurease in CO_2 und NH_3 gespalten wird (Tamiya und Usami (107)).

so ist der Vorgang der Nitratreduktion bilanzmässig durch folgende allgemeine Formel zum Ausdruck zu bringen (Yamagata (129)):



Der hierbei gebildete NH_3 wird sodann restlos vom Pilz assimiliert, wobei derselbe und die C-Quelle, je nachdem ob diese Hyper- oder Hypoquotienten besitzt, entweder nach (3) oder (4) umgesetzt werden. Unter Berücksichtigung der Formeln (3), (4) und (7) können wir ableiten, wie viel CO_2 bei Bildung einer bestimmten Menge Pilzkörper ausgeschieden werden sollte. Wie wir es bei der NH_3 -Kultur getan haben, können wir aus der Menge des gebildeten Pilzkörpers und der Grösse der Atmung und des Wachstums den RQ_{NO_3} -Wert bei Nitratkultur berechnen.* Die so errechneten Werte stimmen wiederum, wie Tabelle IV zeigt, bei fast allen C-Quellen befriedigend mit den wirklich gefundenen überein.

Aus unseren Wachstumsgleichungen können wir ferner die Menge der C-Quelle ausrechnen, die jeweils als "Baustein" zur Bildung des Pilzkörpers zu betrachten ist (Tamiya (100, 101 102)). Bezeichnet man mit λ_C die Menge (in gm.) der nach den betreffenden Gleichungen zur Bildung von 1 gm. Pilzkörper als "Baustein" verwendeten C-Quelle, so fällt dieser Wert je nach den Arten der C-Quelle recht verschieden aus, wie in der zweiten

* Bezeichnet man die Menge (in cc.) der Kohlensäure, die bei Bildung von 1 gm. Pilzkörper wegen Nitratreduktion freigesetzt wird, mit l_{CO_2} , so ist

$$l_{\text{CO}_2} = 44820 [\text{CQ}] \frac{\% \text{N}}{\Omega}$$

Der respiratorische Quotient bei Zugabe des Nitrates lässt sich nach folgenden Formeln ausrechnen:

bei den C-Quellen mit Hyperquotienten:

$$RQ_{\text{NO}_3} = \frac{I_{\text{O}_2} \text{CQ} + (M - M_0)(\lambda_{\text{CO}_2} + l_{\text{CO}_2})}{I_{\text{O}_2}}$$

bei den C-Quellen mit Hypoquotienten:

$$RQ_{\text{NO}_3} = \frac{I_{\text{CO}_2}}{(M - M_0)\lambda_{\text{O}_2} + \frac{[I_{\text{CO}_2} - (M - M_0)l_{\text{CO}_2}]}{\text{CQ}}}$$

TABELLE IV

 RQ_{NO_2} -WERTE ALS FUNKTION DER ATMUNGS- UND WACHSTUMSGRÖSSE

C-Quelle	λ_{CO_2}	Atmungsgrösse (O ₂ -Aufnahme bzw. CO ₂ -Abgabe in cc.)	Wachstumsgrösse (Pilzgewichtszunahme in mg.)	RQ_{NO_2}	
				gef.	ber.
Gluconsäure	0.25	28.0	36.8	1.39	1.54
Stärke	0.20	25.1	24.0	1.28	1.23
Saccharose	0.20	25.7	34.0	1.46	1.32
Mannose	0.21	36.5	66.5	1.57	1.44
Glucose	0.21	30.5	34.8	1.30	1.28
Galactose	0.21	22.2	31.5	1.33	1.34
Arabinose	0.21	32.2	41.0	1.31	1.31
Dioxyaceton	0.21	10.8	6.5	1.19	1.15
Mannit	0.24	23.1	33.0	1.15	1.23
Sorbit	0.24	40.7	53.8	1.17	1.21
Adonit	0.24	37.0	61.0	1.14	1.21
Erythrit	0.23	30.3	34.8	1.08	1.06
Glycerin	0.18	17.9	17.5	0.94	0.95
Äthylalkohol	0.14	18.5	9.0	0.65	0.64

TABELLE V

VERWERTBARKEIT VON VERSCHIEDENEN C-QUELLEN BEI ZUGABE VON NH_4Cl ALS N-QUELLE

C-Quelle	λ_C	Ökonomischer Koeffizient	Verbrauch der C-Quelle in % (bezogen auf Gesamtumsatz)		(Verbrauch zur Atmung) (Verbrauch zum Wachstum)
			zur Atmung	zum Wachstum	
Gluconsäure	1.59	0.40	37	63	0.59
Saccharose	1.27	0.46	42	58	0.72
Mannose	1.31	0.47	38	62	0.61
Glucose	1.31	0.41	46	54	0.85
Galactose	1.31	0.47	38	62	0.61
Arabinose	1.34	0.44	42	58	0.72
Dioxyaceton	1.34	0.30	60	40	1.50
Mannit	1.24	0.54	33	67	0.49
Sorbit	1.24	0.49	39	61	0.64
Adonit	1.24	0.52	36	64	0.56
Erythrit	1.26	0.52	35	65	0.54
Glycerin	1.27	0.48	40	60	0.67
Äthylalkohol	0.95	0.50	53	47	1.13

Spalte der Tabellen V und VI angegeben ist.* Unter Berücksichtigung des λ_C -Wertes kann man aus der Grösse der Atmung und des Wachstums den sogen. ökonomischen Koeffizienten nach W. Pfeffer (73), d.i. das Verhältnis
 (Menge des aufgebauten Pilzkörpers)

(Menge der insgesamt verbrauchten C-Quelle), ausrechnen,† und auch feststellen, welcher Anteil der insgesamt verbrauchten C-Quelle auch unseren Bilanzgleichungen durch Atmung und Wachstum umgesetzt worden ist. (Vergl. Tabelle V und VI.)

Aus den obigen Tabellen ist zu entnehmen, dass der ökonomische Koeffizient sowie der Quotient $\frac{(\text{Verbrauch der C-Quelle zur Atmung})}{(\text{Verbrauch der C-Quelle zum Wachstum})}$ je nach den C-Quellen recht verschieden ausfällt. Kleinere Werte von diesen letzteren Quotienten (0.5–0.8) wurden bei mehrwertigen Alkoholen (Mannit, Adonit, Erythrit u.a.), Hexosen (Mannose, Galactose u.a.) und Gluconsäure gefunden, während bei den meisten Aminosäuren grössere Quotienten (1.2–3.0) beobachtet wurden.

Unter Berücksichtigung der Verbrennungswärme von C-Quellen und der vom Pilzkörper (durchschnittlich 4.5 Kcal. pro 1 gm. Pilztrockengewicht

* Der λ_C -Wert lässt sich durch folgende Formeln berechnen:
 bei den C-Quellen mit Hyperquotienten:

$$\lambda_C = \frac{\nu_C \mathfrak{M}[CQ]}{\Omega n_C \Phi},$$

bei den C-Quellen mit Hypoquotienten:

$$\lambda_C = \frac{\nu_C \mathfrak{M}}{\Omega n_C},$$

worin \mathfrak{M} das Molekulargewicht der C-Quelle bedeutet.

† Bezeichnet man mit I_C die Menge (in gm.) der veratmeten C-Quelle, so ist
 bei den C-Quellen mit Hyperquotienten:

$$I_C = \frac{\mathfrak{M}[CQ] I_{O_2}}{22410 \cdot n_C},$$

bei den C-Quellen mit Hypoquotienten:

$$I_C = \frac{\mathfrak{M} I_{CO_2}}{22410 \cdot n_C}.$$

Der ökonomische Koeffizient ist:

$$\frac{M - M_0}{(M - M_0) \lambda_C + I_C}$$

TABELLE VI
VERWERTBARKEIT VON VERSCHIEDENEN AMINOSÄUREN

Aminosäuren	λ_C	Ökonomischer Koeffizient	Verbrauch in % (bezogen auf Gesamtumsatz)		(Verbrauch zur Atmung) (Verbrauch zum Wachstum)
			zur Atmung	zum Wachstum	
Alanin	1.23	0.21	74	26	2.85
Serin	1.75	0.15	74	26	2.85
Valin	0.92	0.37	66	34	1.94
Leucin	0.86	0.51	56	44	1.27
Isoleucin	0.86	0.53	55	45	1.24
Asparaginsäure	1.84	0.16	71	29	2.45
Phenylalanin	0.75	0.40	71	29	2.45
Histidin	1.29	0.21	73	27	2.70
Prolin	0.91	0.27	75	25	3.00
Tryptophan	0.74	0.53	61	39	1.56
Arginin	1.29	0.19	74	26	2.85

TABELLE VII
ENERGETISCHER WACHSTUMSERTRAG (RUBNER-KOEFFIZIENT) BEI VERSCHIEDENEN C-QUELLEN

C-Quelle	Berechnet nach der Theorie von Tamiya (<i>Aspergillus oryzae</i>)	Ermittelt von anderen Forschern		
			Organismus	Autor
Saccharose	0.57	0.52	<i>Aspergillus niger</i>	Raulin (78)*
		0.59		De Caro (10)
		0.51		Molliard (55)
Glucose	0.52	0.56	<i>Aspergillus niger</i>	Terroine u.a. (116)
Glycerin	0.54	0.58	<i>Aspergillus niger</i>	De Caro (10)
		0.58	<i>Aspergillus niger</i>	Terroine u.a. (112, 113)
Äthylalkohol	0.33	0.30	<i>Eurotiopsis Gayoni</i>	Laborde (51)*
Alanin	0.20	0.39	<i>Aspergillus niger</i>	Terroine u.a. (114, 115)
Valin	0.28	0.39	<i>Aspergillus niger</i>	Terroine u.a. (114, 115)
Leucin	0.40	0.38	<i>Aspergillus niger</i>	Terroine u.a. (114, 115)

* Berechnet von W. Kruse (50) nach dem Experiment des genannten Autors.

nach Yamamoto und Endo (133), Vergl. Kapitel IV) können wir aus dem ökonomischen Koeffizienten auch den von M. Rubner vorgeschlagenen Massstab des energetischen Ausnutzungsgrades des Wachstumsvorgangs, d.i.

das Verhältnis $\frac{(\text{Verbrennungswärme des gebildeten Pilzkörpers})}{(\text{Verbrennungswärme der insgesamt verbrauchten C-Quelle})}$,
ermitteln. Wie Tabelle VII zeigt, stimmen die von uns indirekt aus der Atmungs- und der Wachstumsgrösse berechneten Rubner-Koeffizienten im Grossen und Ganzen mit den früher von anderen Forschern ermittelten Werten überein. Die Abweichungen sind auf die Verschiedenheit der gebrauchten Pilzstämme sowie der Kulturbedingungen, insbesondere aber wohl auf die Verschiedenheit der Kulturdauer, zurückzuführen. Auf die Erörterung der Veränderung des Wachstumsertrages kommen wir später zurück.

Auch für den Fall, wo als N-Quelle Nitrat in Betracht kommt, können wir den ökonomischen Koeffizienten auf indirektem Wege aus der Grösse von Atmung und Wachstum unter Berücksichtigung des zusätzlichen Umsatzes durch Nitratreduktion (7) ausrechnen.* Wie aus Tabelle VIII ersichtlich, ergeben sich bei den Nitratkulturen stets kleinere ökonomische Koeffizienten als bei den entsprechenden NH_3 -Kulturen. Der auf die Nitratreduktion entfallende Umsatz fällt bei verschiedenen C-Quellen ziemlich konstant, und zwar gegen 5–11% vom ganzen Umsatz, aus. Der Quotient $\frac{(\text{Umsatz zur Atmung})}{(\text{Umsatz zum Wachstum})}$ fällt bei einigen C-Quellen (verschiedenen Zuckerarten und meisten mehrwertigen Alkoholen) fast ebenso gross,

* Bezeichnen wir mit l_c die Menge (in gm.) der C-Quelle, die bei Bildung von 1 gm. Pilzkörper nach der Gleichung (7) zur Reduktion von Nitrat verbraucht wird, so ist

$$l_c = \frac{m_{\text{CO}_2}}{22410 \cdot n_c} = \frac{2m_{\text{N}}[CQ]}{n_c \Omega}$$

Im Fall der Nitratkultur muss die Menge der veratmeten C-Quelle, l_c , nach folgenden Formeln berechnet werden:

bei den C-Quellen mit Hyperquotienten:

$$l_c = \frac{m_{\text{O}_2}[CQ]}{22410 \cdot n_c},$$

bei den C-Quellen mit Hypoquotienten:

$$l_c = \frac{m[I_{\text{CO}_2} - (M - M_0)l_{\text{CO}_2}]}{22410 \cdot n_c}.$$

Der ökonomische Koeffizient ist dabei:

$$\frac{M - M_0}{l_c + (M - M_0)(\lambda_c + l_c)}.$$

bei anderen C-Quellen (Gluconsäure, Äthylalkohol, Erythrit und Glycerin) bedeutend grösser aus wie die Werte bei entsprechenden NH_3 -Kulturen. Diese Tatsache deutet darauf hin, dass bei den letzteren C-Quellen der Vorgang des Wachstums, einschliesslich der Reaktion der Nitratreduktion, mit grösseren Mengen des Atmungsumsatzes "verknüpft" ist als bei den entsprechenden NH_3 -Kulturen. Auf die Frage der "Verknüpfung" zwischen Wachstum und Atmung gedenke ich im Kapitel V näher zurückzukommen.

TABELLE VIII

VERWERTBARKEIT VON VERSCHIEDENEN C-QUELLEN BEI ZUGABE VON KNO_3 ALS N-QUELLE

C-Quelle	Ökonomischer Koeffizient	Verbrauch der C-Quelle in % (bezogen auf Gesamtumsatz)			(Verbrauch zur Atmung) (Verbrauch zum Wachstum)
		zur Atmung	zum Wachstum	zur Nitratreduktion	
Gluconsäure	0.33	40	52	8	0.77
Saccharose	0.41	39	52	9	0.75
Glucose	0.37	43	48	9	0.89
Mannose	0.44	33	58	9	0.57
Galactose	0.41	39	54	7	0.72
Arabinose	0.38	40	51	9	0.78
Dioxyaceton	0.27	59	35	6	1.68
Mannit	0.46	32	57	11	0.56
Sorbit	0.44	35	55	10	0.64
Adonit	0.48	29	60	11	0.48
Erythrit	0.41	39	52	9	0.75
Glycerin	0.37	44	47	9	0.94
Äthylalkohol	0.32	65	30	5	2.17

Schon an dieser Stelle sei aber ausdrücklich darauf hingewiesen, dass die oben angeführten Bilanzgleichungen des Wachstumsvorgangs sowie der Nitratreduktion keineswegs den wahren Chemismus der in Betracht kommenden Zellvorgänge wiedergeben, sondern dass sie nur formale bilanzmässige Ausdrücke derselben darstellen, womit also gar nichts ausgesagt ist, ob und durch welchen Mechanismus solche Zellvorgänge tatsächlich vollzogen werden. Man darf nicht annehmen, dass in den Zellen das Wachstum sowie die Nitratreduktion wirklich nach den angeführten Bilanzgleichungen etwa unabhängig von einander sowie getrennt von Atmungs Vorgängen stattfinden. Auch dieses Problem soll erst in einem späteren Kapitel ausführlicher erörtert werden.

IV. Wärmebilanz des Wachstums

Schon von alters her ist in Japan eine besondere Redensart im Gebrauch, durch welche interessanterweise eine wesentliche physiologische Tätigkeit von *Aspergillus oryzae* bündig zum Ausdruck gebracht wird. An schwülen Tagen oder in einem dumpfen Zimmer sagt man oft: "heiss und feucht wie in einem Koji-Muro!" "Koji-Muro" bedeutet das Lager oder den Keller, in welchem Koji, Massenkultur von *Aspergillus oryzae* auf Reis, zum Reifen gebracht wird. So ist es uns schon lange bewusst, dass der Pilzwuchs mit einer beträchtlichen Wärmeentwicklung verbunden ist, ohne dass man jedoch nach der Ursache dieser merkwürdigen Erscheinung gefragt hätte. Seit dem man aber unterrichtet ist, dass alle Organismen durch ihre Atmung oder Gärung Wärme entwickeln, wurde die betreffende Erscheinung als Folge der durch die intensive Atmung von *Aspergillus* hervorgerufenen Wärmeabgabe aufgefasst.

Die Feststellung, dass der normale Stoffwechsel von *Aspergillus* nicht bloss aus "Atmung," sondern auch zum beträchtlichen Teil aus dem durch unsere Bilanzgleichungen (3, 4) u.a. aufgezeigten Stoffumsatz besteht, hat uns den Gedanken nahegelegt, dass auch dieser letztere Prozess zum Wärmebildungsvorgang des Pilzes, sei es im positiven oder im negativen Sinne, beitragen müsste. Früher herrschte in der Lehre der Bioenergetik ganz allgemein die Anschauung, dass der Vorgang des Körperaufbaues aus dessen Bausteinen als ganzes eine endotherme Reaktion darstelle, wonach also angenommen werden musste, dass bei der kräftig wachsenden Pilzkultur die Menge der insgesamt abgegebenen Wärme um einen gewissen Betrag kleiner sei als man sie nach der Grösse der Atmung rechnerisch hätte erwarten können. Ausgehend aus der oben dargelegten Bilanzanalyse des Pilzstoffwechsels kamen wir nun, im schroffen Gegensatz zu der bisherigen Ansicht, zu dem Schluss, dass der Wachstumsvorgang an und für sich eine exotherme Reaktion darstellt, und zwar aus folgenden Gründen (Tamiya (100, 101, 102)).

Wie schon erwähnt, können wir nach unseren Bilanzformeln (3) und (4) diejenige Menge der C-Quelle ausrechnen, die man als "Baustein" bei der Bildung des Pilzkörpers betrachten darf. In Tabelle IX sind in der Rubrik λ_C die Menge der auf 1 gm. Pilzkörper bezogenen "Baustein-C-Quelle," und daneben dessen Verbrennungswärme angegeben, welche nach den thermochemischen Daten ausgerechnet wurde. Zum Vergleich mit solchen kalorischen Werten wurde die Verbrennungswärme der Mycelien von *Aspergillus*—wie üblich unter Anwendung eines Bombenkalorimeters nach Berthelot—ermittelt, wobei sich der Wert 4.45–4.60 (Durchschnitt:

4.5) Kcal. pro gm. Pilztrockengewicht ergab (Yamamoto und Endo (133)). Wie ersichtlich, ist der Energiegehalt von λ_C gm. C-Quelle in allen Fällen gewissermassen grösser als die Verbrennungswärme von 1 gm. Pilzkörper, was schon darauf hindeutet, dass die durch die Bilanzgleichungen (3) und (4) ausgedrückten Umsätze exotherme Reaktionen darstellen. Unter Berücksichtigung des Tatbestandes, dass bei der Verbrennung vom Pilzkörper die N-Atome bis zur Stufe von Nitrat oxydiert werden, und ferner dass bei der NH_3 -Kultur eine gewisse Wärmefaufnahme bei der Entbindung von NH_3 aus dessen Salzen, bei Kultur auf Aminosäuren hingegen eine Wärmeabgabe wegen der Neutralisation des abgespaltenen NH_3 durch Kohlensäure stattfinden sollte, wurde für einzelne C-Quellen die Wärmetönung des Wachstumsvorgangs theoretisch ausgerechnet. In der letzten Spalte der Tabelle IX sind die betreffenden Werte pro gm. Pilztrockengewicht angegeben; ausnahmslos stellten sich bei allen C-Quellen positive Werte heraus, deren Grösse allerdings von Fall zu Fall ziemlich weitgehend verschieden ist.

TABELLE IX
DER WÄRMEGEHALT VON "BAUSTEIN-C-QUELLE" UND DER THEORETISCHE WERT DER
"WACHSTUMSWÄRME" PRO BILDUNG VON 1 GM. PILZKÖRPER

C-Quelle	λ_C	Wärmegehalt der Baustein-C-Quelle (Kcal.)	Wachstumswärme pro Bildung von 1 gm. Pilzkörper (Kcal.)
Glucose	1.31	4.9	+0.7
Galactose	1.31	4.9	+0.7
Mannit	1.24	5.0	+0.9
Glycerin	1.27	5.5	+1.3
Äthylalkohol	0.95	6.7	+2.5
Valin	0.92	5.5	+0.9
Alanin	1.23	5.4	+0.3
Leucin	0.86	5.6	+1.1

Zur Prüfung dieser Folgerung wurden folgende Versuche ausgeführt (Yamamoto und Yamagata (134)). In einem Mikrokalorimeter, das gleichzeitig als Respirometer dienen konnte (Tamiya und Yamamoto (109)), wurde eine Kultur von *Aspergillus oryzae* angestellt (vergl. Fig. 1). Die Nährlösung enthielt Galactose und NH_4Cl als C- bzw. N-Quelle. Während der Kultur, die 75 Stunden dauerte, wurden insgesamt 311.6 cal. Wärme abgegeben, 54.7 cc. Sauerstoff durch Atmung verbraucht, und 55 mg. Pilzkörper (Trockengewicht) neugebildet (vergl. Fig. 2). Aus der Atmungsgrösse und der Verbrennungswärme von Galactose wurde die Atmungswärme zu 276.8 cal. berechnet; die Differenz $311.6 - 276.8 = 34.8$ cal. muss also auf den "Wachstumsvorgang" zurückgeführt werden. Die auf Bildung von 1 gm. Pilzkörper bezogene Wachstumswärme beträgt danach 0.633 Kcal., ein Wert, der innerhalb der Fehlergrenze vorzüglich mit dem theoretischen Wert 0.7 Kcal. übereinstimmt.

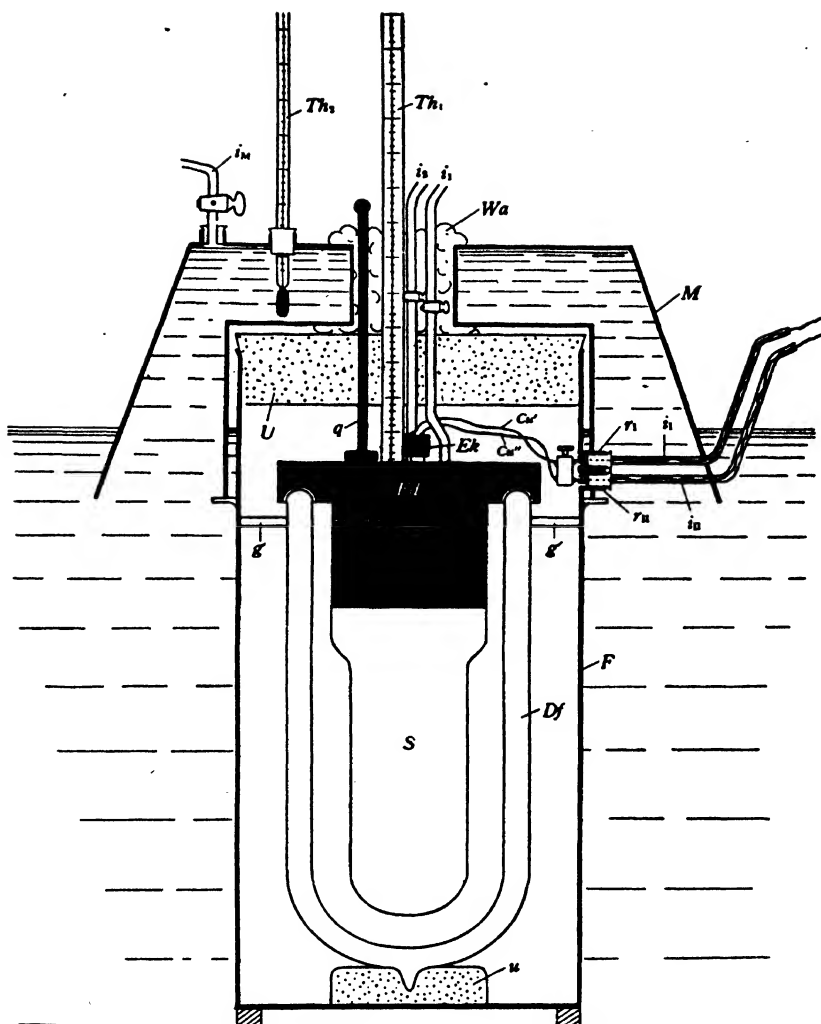


Fig. 1a.

Figs. 1a und b.—Mikrokalorimeter nach Tamiya und Yamamoto (109).

K, metallene Kulturbombe; S, Behälter des Kalorimeterwassers; L, halbkugelförmiger Becher, worin der Pilz zur Entwicklung gebracht wird; J, Rührer des Kalorimeterwassers; Th, Beckmann-Thermometer; i_1 und i_2 , Glasröhrchen, die zur Gasleitung sowie zur Gaswechselformung dienen; Ed, Ebonitdeckel; Df, Dewar-Flasche; M, doppelwandiger Metallmantel, worin das Thermostatenwasser eingefüllt werden kann; i_1 und i_2 , elektrische Leitungsdrähte, wodurch der in L befindliche Manganindraht (22.87 Ohm) erhitzt werden kann. Durch regulierte Wärmeerzeugung an diesem Manganindraht wurde die Eichung des Kalorimeters gewährleistet.

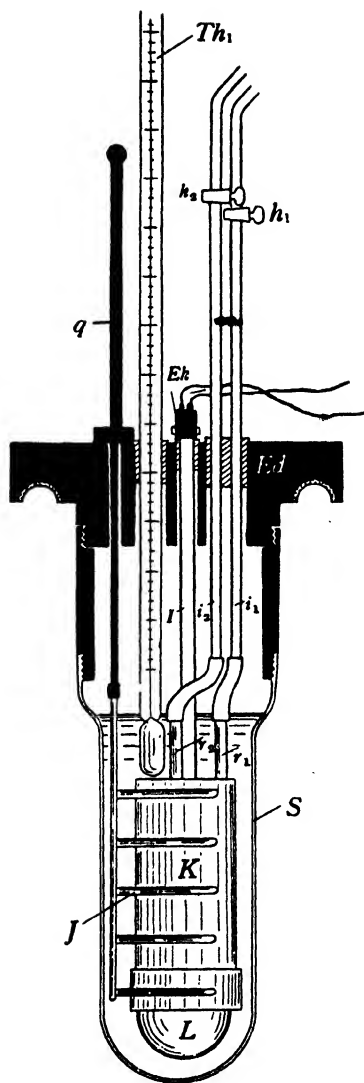


Fig. 1b.

Einfacher aber weniger exakt kann die Wachstumswärme auch auf folgende Weise ermittelt werden. Bezeichnet man mit

U_M = die Verbrennungswärme des gebildeten Pilzkörpers,

U_W = die insgesamt abgegebene Wärme,

U_1 und U_2 = die Verbrennungswärme des Nährmediums am Anfang bzw. am Ende des Versuchs, so muss nach dem Erhaltungsprinzip der Energie folgende Beziehung bestehen:*

$$U_1 - U_2 = U_M + U_W \quad (8)$$

Nach den Ergebnissen unserer Bilanzanalyse können wir andererseits folgende Formel aufstellen:

$$U_1 - U_2 = U_R + U_B \quad (9)$$

wobei U_R = die durch Atmung freigesetzte Wärme,

U_B = die Verbrennungswärme der "Baustein-C-Quelle"

bedeutet. Aus (8) und (9) lassen sich ableiten:

$$U_B - U_M = (U_1 - U_2) - U_M - U_R$$

Die Wachstumswärme $U_B - U_M$ kann also dadurch ermittelt werden, dass man die Grösse von U_1 , U_2 , U_M und U_R bestimmt, wofür letztere aus der Grösse der O_2 -Aufnahme (bei den C-Quellen mit Hyperquotienten) berechnet werden kann. Die mit *Aspergillus melleus* und *Aspergillus oryzae* unter Zugabe von Glucose bzw. Mannit

* Dass diese Gleichung bei der Kultur von *Aspergillus niger* mit der Annäherung von 98% gültig ist, ist von L. Algera (1) bewiesen worden.

(ebenfalls mit NH_4Cl als N-Quelle) ausgeführten Versuche ergaben folgende Resultate (vergl. Tabelle X, Yamamoto und Endo (133)).

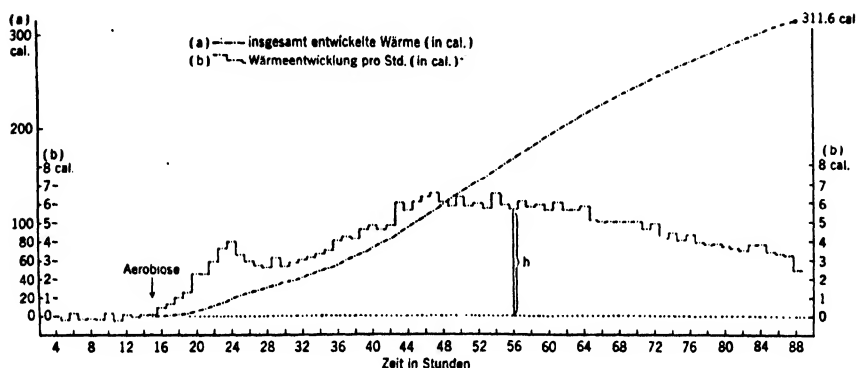


Fig. 2.—Die Wärmeabgabe bei Kultur von *Aspergillus oryzae* auf Galactose (Yamamoto und Yamagata (134)).

* Die Höhe der Kurve (z.B. h) zeigt die Wärmemenge, welche in einer Stunde, die 30 Minuten vor und nach jedem Zeitpunkt umfasst, abgegeben wurde.

TABELLE X

WÄRMEBILANZ DES WACHSTUMS BEI *Aspergillus oryzae* UND *Aspergillus melleus* (ERKLÄRUNG IM TEXT)

<i>Aspergillus</i>	C-Quelle	Wachstums- grösse (Zunahme des Pilztrockengew. in gm.): M	U_1 (Kcal.)	U_2 (Kcal.)	U_M (Kcal.)	U_R (Kcal.)	$U_B - U_M$ (Kcal.)	$\frac{U_B - U_M}{M}$ (Kcal.)
<i>Oryzae</i>	Mannit	1.274	28.54	13.74	5.67	6.81	2.32	+1.82
<i>Melleus</i>	Glucose	0.578	24.81	17.76	2.76	3.32	0.96	+1.66

Die auf 1 gm. Pilz bezogene Wachstumswärme beträgt in diesem Fall 1.66 Kcal. bei Glucose und 1.82 Kcal. bei Mannit. Die Übereinstimmung mit den theoretisch abgeleiteten Werten (0.7 bzw. 0.9 Kcal.) war hierbei weniger gut, doch mag es jedenfalls als bewiesen gelten, dass der "Wachstumsvorgang" an und für sich eine exotherme Reaktion darstellt.

Nach den in Tabelle IX vorgelegten theoretischen Zahlen ist zu erwarten, dass z.B. bei Äthylalkohol und Glycerin eine viel grössere Menge der Wachstumswärme erzeugt wird, als es bei Hexose oder Mannit der Fall ist. Ferner würde die Wachstumswärme bei der Nitratkultur grösser ausfallen als bei der entsprechenden NH_3 -Kultur, weil auch der Vorgang (7) an und für sich eine exotherme Reaktion sein muss.

In dem Koji-Muro kommen als C-Quelle fast ausschliesslich Kohlehydrate und als N-Quelle in der Hauptsache Nitrat in Betracht. Auf Grund unserer kalorimetrischen Messungen darf man sagen, dass in dem Koji-Muro eine um wenigstens mehr als 10% grössere Menge Wärme produziert wird als es, wie früher angenommen wurde, der Atmung des Pilzes entspricht. Auch ein anderes Attribut des Koji-Muro, nämlich die ungeheure Feuchtigkeit, kann nicht, wie allgemein angenommen, ausschliesslich auf die Wasserproduktion durch Atmung zurückgeführt werden, sondern es muss dabei z.T. auch durch den Wachstumsvorgang, gemäss unseren Bilanzgleichungen (3, 4) u.a. eine gewisse Menge Wasser freigesetzt werden.

V. Aufbau- und Erhaltungsatmung

In den vorangehenden Kapiteln haben wir dargetan, dass der Stoffwechsel von *Aspergillus* insofern er vom bilanzanalytischen Standpunkt aus betrachtet wird, sich säuberlich in zwei Teile: "Atmung" und "Wachstum" zerlegen lässt. Damit ist aber keineswegs etwas darüber ausgesagt, dass diese Hauptzüge des physiologischen Umsatzes auch in ihren inneren Mechanismen von einander getrennt seien. Durchaus wahrscheinlich, ja wohl offensichtlich stehen diese beiden Prozesse in ihren inneren Mechanismen ineinandergreifend und eng verflochten zueinander in gegenseitiger Wechselwirkung. Diese Unterscheidung hat, wie wir es oben getan haben, zunächst nur in dem Sinne Bedeutung, dass dadurch die sonst totalen und durchaus kompakt erscheinenden physiologischen Phänomene äusserlich, aber doch jedenfalls quantitativ nach den experimentell erfassbaren Merkmalen in ihre Teile zergliedert werden können.

Hat man eine innere Verbindung des Wachstumsvorgangs mit der Atmung anzunehmen, so muss andererseits daran gedacht werden, dass es auch einen bestimmten Anteil der Atmung gibt, die nichts direkt mit dem Wachstum zu tun hat, sondern bloss mit der "Erhaltung" des existierenden Organismenkörpers verbunden ist. Dass das Lebewesen, auch wenn es nicht positiv wächst, noch immer "atmet," und dass die "Erhaltung" des Lebens durch Sistierung der Atmung bald unmöglich gemacht wird, ist eine allbekannte Tatsache.

Wir wollen bei unserer analytischen Betrachtung einen Schritt weiter gehen und fragen, ob und wie viel Anteil der Atmung mit dem Wachstum einerseits und mit der Erhaltung andererseits verbunden sei. Um diese Frage beantworten zu können, haben Tamiya und Yamagutchi (108) folgenden Versuch angestellt.

An einer Kultur von *Aspergillus melleus* auf Glucose (NH_4Cl als N-Quelle) wurde der zeitliche Verlauf der Atmung und des Wachstums messend verfolgt (Kulturdauer = 110–138 Stdn.). Durch Analyse der zeitlichen Kurve der Atmungs- und der Wachstumsgrösse wurden bezüglich verschiedener Zeitpunkte der Kultur folgende Werte ausgerechnet:

μ = die Menge (in gm.) des von 1 gm. Pilz in einer Stunde neugebildeten Pilzkörpers,
 Q_{O_2} = die von 1 gm. Pilz in einer Stunde bewirkte Atmung (Menge des aufgenommenen Sauerstoffs in cc.).

Nun könnte man nach der obigen Überlegung folgende Formel aufstellen:

$$Q_{O_2} = \mu \lambda_a + Q_e \quad (10)$$

worin λ_a = die Menge der Atmung (O_2 -Menge in cc.), die mit der Bildung von einer Gewichtseinheit (1 gm.) des Pilzkörpers quantitativ verbunden sein sollte,
 Q_e = diejenige Grösse der Atmung, welche mit der 1-stündigen Erhaltung von 1 gm. Pilzkörper verknüpft ist,

bedeutet. Die Frage nach der quantitativen Verkettung der Atmung mit dem Wachstumsvorgang kann durch Feststellung der Grösse von λ_a beantwortet werden. Wenn die Atmung gar nicht mit dem Wachstumsvorgang, wohl aber ausschliesslich mit der Erhaltung verbunden wäre, so müsste es lauten: $\lambda_a = 0$ und $Q_e = Q_{O_2}$.

Mit den zahlreichen Q_{O_2} - und μ -Werten, die in verschiedenen Kulturstadien erhalten wurden, wurden mehrere der Formel (10) entsprechende Gleichungen aufgestellt, und aus diesen haben wir mit Hilfe der Methode der kleinsten Quadrate die durchschnittlichen Grössen der beiden unbekannten Werte errechnet, wobei in den zweimal angestellten Versuchen folgende Ziffern erhalten wurden:

Versuch I = $\lambda_a = 220$, $Q_e = 14$

Versuch II = $\lambda_a = 260$, $Q_e = 14$

Die innige quantitative Verknüpfung zwischen Wachstum und Atmung gilt also bewiesen. In dieser Ausführung bleibt aber noch unentschieden, ob die Grösse der "Aufbauatmung" (λ_a) und der "Erhaltungsatmung" (Q_e) während der ganzen Kulturdauer durchaus konstant ausfällt oder nicht. Die Antwort auf diese Frage gaben uns nun folgende Betrachtungen:

Unter Anwendung von verschiedenen Q_{O_2} und μ -Werten, die durch Analyse der Kurvendarstellung der Atmung bzw. des Wachstums erhalten wurden, haben wir bezüglich jedes aufeinanderfolgenden Zeitpunktes die Gleichungen nach (10) aufgestellt. Die Gleichungen, die sich auf zwei unmittelbar aufeinanderfolgende Kulturzeiten beziehen, wurden als simultane Gleichungen angesehen und aus diesen wurde die Grösse von λ_a und Q_e berechnet, unter der Annahme, dass die betreffenden Werte während der in Betracht kommenden kurzen Zeitspanne konstant blieben. Auffallenderweise ergaben sich aus verschiedenen Paarungen der Gleichungen verschiedene Werte von λ_a und Q_e , wie sie in Spalte 5 und 8 der Tabelle XI wiedergegeben sind.

TABELLE XI

ZEITLICHE VERÄNDERUNG VON AUFBAU- UND ERHALTUNGSATMUNG (ERKLÄRUNG IM TEXT)

Zeit in Stdn.	Wachstums- grösse μ	Atmungsgrösse		Aufbaumatmung			Erhaltungs- atmung		Gesamt- umsatz Q_{ZC}
		Q_{O_2}	Q_C	λ_a	λ_A	$\mu\lambda_A$	Q_c	Q_E	
54-55	0.065	28.8	0.039	149	0.20	0.013	19.1	0.026	0.124
58-59	0.048	26.0	0.035	155	0.21	0.010	18.6	0.025	0.100
66-67	0.028	21.9	0.029	242	0.32	0.009	15.2	0.020	0.066
74-75	0.016	18.6	0.025	318	0.43	0.007	13.6	0.018	0.046
82-83	0.006	15.3	0.020	384	0.51	0.003	12.9	0.017	0.029
86-87	0.002	13.5	0.018	411	0.55	0.001	12.8	0.017	0.020

Wir stehen hier einer recht interessanten Tatsache gegenüber, dass nämlich mit dem Altern des Pilzes der Wert λ_a immer grösser, der Wert Q_c dagegen allmählich kleiner wird. Der Vorgang des Körperaufbaues ist also bei alternden Pilzen mit kräftigerer Atmung verbunden als bei den jüngeren, wogegen die Erhaltung bei älteren Pilzkörpern mit geringerer Atmung verknüpft ist als bei den jüngeren.

In der Tabelle XI bedeuten λ_A und Q_E die Grösse der Aufbau- bzw. der Erhaltungsatmung, ausgedrückt nach der Menge (in gm.) des veratmeten Substrates (in diesem Fall Glucose). Bezeichnet man nun mit

Q_C = die Menge (gm.) der in einer Stunde von 1 gm. Pilz veratmeten C-Quelle,

Q_{ZC} = die Menge der in einer Stunde von 1 gm. Pilz insgesamt verbrauchten C-Quelle,

so ist, da pro Bildung von μ gm. Pilz $\mu\lambda_C$ gr. C-Quelle als "Baustein" verbraucht werden sollen, folgende Formel aufzustellen:

$$Q_C = \mu\lambda_A + Q_E \quad (11)$$

$$\begin{aligned} Q_{ZC} &= \mu\lambda_C + Q_C \\ &= \mu(\lambda_C + \lambda_A) + Q_E \end{aligned} \quad (12)$$

In der Tabelle XII sind die auf den Gesamtumsatz Q_{ZC} bezogenen Prozentsätze von μ , $\mu\lambda_C$, $\mu\lambda_A$, Q_E und Q_C angegeben.

Daraus ergibt sich zunächst, dass der ökonomische Koeffizient (μ/Q_{ZC}), wie alle übrigen in Betracht kommenden Werte, je nach dem Alter des Pilzes veränderlich ist; er ist nämlich umso grösser, je jünger und wachstumsfähiger der Pilz ist, und mit dem Altern des Pilzes sinkt er allmählich ab, um schliesslich null zu werden. Parallel damit verändert sich auch die Menge der als "Baustein" verwendeten C-Quelle ($\mu\lambda_C$), die bei jüngeren Pilzkörpern sogar etwa 70% vom ganzen Verbrauch der C-Quelle

TABELLE XII

ZEITLICHE VERÄNDERUNG VON WACHSTUMS- UND ATMUNGSUMSATZ IN % BEZOGEN AUF GESAMTUMSATZ ($Q_{\Sigma C}$)

Zeit in Std.	Wachstum μ (Ökonomi- scher Koeffi- zient $\times 100$)	Baustein $\mu\lambda_C$	Aufbau- atmung $\mu\lambda_A$	Erhaltungsatmung Q_E	Gesamtatmung Q_C
54-55	53	69	10	21	31
58-59	50	65	10	25	35
66-67	42	55	14	31	45
74-75	34	45	15	40	55
82-83	21	28	11	61	72
86-87	8	11	4	85	89
94-104	0	0	0	100	100

ausmacht, bei späteren Kulturstadien aber bis auf null herabsinkt. In früheren Kulturstadien wird ungefähr $\frac{1}{3}$ der gesamten Atmung zum Aufbau- und Stoffwechsel und die übrigen $\frac{2}{3}$ zum Erhaltungsstoffwechsel verwandt, während dieses Verhältnis mit der Zeit allmählich zugunsten der Erhaltungsatmung zunimmt, bis schliesslich bei späteren Kulturstadien tatsächlich 100% der gesamten Atmung auf Erhaltungsstoffwechsel bezogen wird. Was den insgesamt auf Wachstum bezogenen Umsatz ($\mu\lambda_C + \mu\lambda_A$) anbelangt, so beträgt er bei früheren Kulturstadien ca. 80% von dem gesamten Umsatz; auch dieser Prozentsatz sinkt aber mit dem Fortschreiten der Kultur ziemlich schnell ab, um schliesslich bei etwa 100 Kulturstunden null zu werden.

Wie dürfte nun die Tatsache zu deuten sein, dass die Werte λ_A und Q_E sich je nach dem Alter oder der Aktivität der Zellen in umgekehrter Richtung verändern? Man könnte sagen, dass die Aktivität oder Jugend die Labilität oder Empfindlichkeit des lebenden Systems, während Inaktivität oder Alter die Stabilität oder Trägheit desselben bedeuten. Mit der in der Thermodynamik gebräuchlichen Terminologie könnte man auch sagen, die jüngeren und aktiveren Zellen seien in unwahrscheinlicherem, mehr geordnetem Zustand als die älteren. Dass zur Erhaltung der jüngeren Zellen auf ihrem labileren und mehr geordneten Zustand mehr Aufwand des stofflichen sowie energetischen Umsatzes vonnöten ist als zur Erhaltung der älteren Zellen, d. i. Q_C oder Q_E um so grösser ausfallen je jünger die Zellen sind, ist also grundsätzlich nicht schwer verständlich.

Einige besondere Bemerkungen beanspruchen dagegen die physiologische Bedeutung von λ_A und seiner zeitlichen Veränderung. Zur Bildung von 1 gm. Pilzkörper ist insgesamt $\lambda_C + \lambda_A$ gm. C-Quelle notwendig, wovon—die Konstanz der Zusammensetzung des Pilzkörpers vorausgesetzt—formell

nur λ_A mit der Zeit veränderlich ist. Nun ist der Vorgang des Wachstums, wie kompliziert auch sein innerer Mechanismus sowie die chemische Struktur des gebildeten Pilzkörpers sein mögen, doch in Bausch und Bogen etwa mit der organischen Synthese im chemischen Laboratorium vergleichbar. Es muss nämlich eine bestimmte Menge des Ausgangs- oder Rohmaterials verwendet und verarbeitet werden, wobei mit der erzielten Synthese stets ein gewisser materieller Verlust oder Entstehung der "Späne" unvermeidlich ist.

Hier sei die früher von uns aufgestellte formale Bilanzformel des Wachstumsvorgangs vereinfacht wie folgt umgeschrieben:



worin B_0 entweder "Baustein-C-Quelle" plus "Baustein-Sauerstoff" (bei den C-Quellen mit Hypoquotienten) oder "Baustein-C-Quelle" allein (bei den C-Quellen mit Hyperquotienten), und A_0 entweder "Späne-Kohlensäure" plus "-Wasser" (bei den C-Quellen mit Hyperquotienten) oder "Späne-Wasser" allein (bei den C-Quellen mit Hypoquotienten) bedeutet. Diese Formel, die wir deduktiv unter Berücksichtigung der elementaren Zusammensetzung des Pilzkörpers und der der C-Quelle abgeleitet haben, gibt den Grenzfall des Umsatzes wieder, der erst dann realisiert werden könnte, wenn der Aufbauprozess mit minimalstem Verlust, also mit grösstmöglicher materieller Ausbeute ideal vollzogen würde. Offenbar kann der chemische Prozess der Körpersynthese kaum ideal erfolgen, sodass tatsächlich grössere Mengen der Rohmaterialien verbraucht und grössere Mengen der "Späne"-Substanzen ausgeschieden werden müssen als sie der Formel (13) entsprechen. Übrigens muss als Rohmaterial ausser C-Quellen—auch bei den C-Quellen mit Hyperquotienten—stets O_2 , und als "Späne"-Substanzen ausser Wasser—auch bei den C-Quellen mit Hypoquotienten—stets CO_2 in Betracht gezogen werden. Wollen wir den wahren chemischen Umsatz beim Pilzkörperaufbau wie folgt zum Ausdruck bringen:



so müssen B und A stets grösser als B_0 bzw. A_0 sein, während $n = n_0$ sein soll, denn wir wissen, dass beim Wachstum die Gesamtmenge des verbrauchten NH_3 ohne jeglichen Verlust im Pilzkörper assimiliert wird.

Die Differenz zwischen (13) und (14):

$$(B - B_0) = (A - A_0)$$

ist der Umsatz, den wir in unserer formalen Bilanzanalyse als "Atmung" erkannt haben. Dieser Anteil der Atmung, der als "Späneatmung" des Wachstums bezeichnet werden mag, stellt wenigstens einen Teil der "Aufbauatmung" dar, weil sie stets notwendig mit dem Wachstum verbunden stattfindet.

Nun muss es aber andererseits einen gewissen Teil der Atmung geben, die ohne in der Formel (14) einbegriffen zu sein, doch quantitativ mit dem Vorgang der Pilzkörpersynthese verbunden ist, und zwar in dem Sinne, dass sie den Wachstumsvorgang ermöglicht als Energielieferant für mannig-

faltige chemische und physikalische Arbeiten, die in verschiedenen Teilprozessen der Synthese geleistet werden müssen. Dass für die Erzielung der Körpersynthese nicht nur chemische sondern vielfach auch physikalische Arbeit aller Art geleistet werden muss, sei es zur Verwirklichung allerlei günstiger Konstellationen des plasmatischen Systems, sei es zur Beseitigung allerlei Arten von Hemmnissen u.s.w., liegt wohl auf der Hand. Derartig Arbeit könnte zwar vielfach automatisch durch die energieliefernden Teilreaktionen in dem Umsatz (14) selbst geleistet werden, aber allem Anschein nach dürfte Arbeit auch noch auf Kosten der Energie der Atmung geleistet werden, die in dem durch die Formel (14) ausgedrückten Umsatz nicht einbegriffen ist. Dies wäre mit der Arbeit zu vergleichen, die bei organischer Synthese im Laboratorium vom Chemiker selbst geleistet wird. Ohne Arbeit des Chemikers können überhaupt keine zielklaren Reaktionen, selbst wenn sie thermodynamisch möglich sind, realisiert werden.

Ob und welcher Anteil der ganzen Aufbauatmung aus der energieliefernden Atmung in diesem Sinne einerseits und aus der Späneatmung andererseits besteht, darüber können wir gar nicht Bestimmtes aussagen. Auf jeden Fall ist die zeitliche Veränderung von λ_A dahin zu deuten, dass mit dem Altern des Pilzes sowohl die Späneatmung wie auch die Atmung zur besprochenen Arbeitsleistung vergrößert wird. Die Vergrößerung der Späneatmung bedeutet die Verminderung der Ausbeute oder die Verschlechterung der materiellen Ökonomie bei der chemischen Prozedur der Synthese. Während die Vergrößerung der Atmung zur Arbeitsleistung die Vermehrung des Energieaufwandes bei verschiedenen Arbeiten, die zur Realisierung von allerlei für den Aufbauprozess notwendigen Bedingungen geleistet werden müssen, bedeutet. Alles ist also als Folge der Entartung des "Aktivitätszustandes" der Zellen zu verstehen, die mit dem Älterwerden der Zelle allmählich eintreten muss.

Die Menge der C-Quelle, die bei der chemischen Synthese des Pilzkörpers tatsächlich als Baustein verwendet wird, entspricht λ_C plus dem Umsatz durch die Späneatmung, und zwar fällt sie zwischen die Grenzen λ_C und $\lambda_C + \lambda_A$. Kommt der Zucker als C-Quelle in Betracht, so beträgt selbst dieser grösste Wert $\lambda_C + \lambda_A$ bei jüngerem Pilz 1.5 und bei gealtertem Pilz 2.0. Dass ein kleines Pilzchen aus nur 15–20 gm. Zucker 10 gm. Zellsubstanz mit allen Varietäten und aller Kompliziertheit der Bestandteile zu synthetisieren vermag, ist eine wunderbare Tatsache, die freilich aller menschlichen Leistungen spottet.

Was wir über die Bedeutung der Bilanzgleichung des Wachstums gesagt haben, gilt *mutatis mutandis* auch für diejenige der Nitratreduktion. Die

Gleichung (7) gibt weder den wahren Chemismus der Nitratreduktion wieder, noch bedeutet sie, dass die Nitratreduktion etwa unabhängig vom Wachstumsvorgang bzw. von der Atmung selbständig stattfinden könnte. Ohne Zweifel wird die Reduktion von Nitrat primär nicht—wie durch unsere Bilanzgleichung (7) ausgedrückt wurde—durch Koppelung mit der Oxydation der C-Quelle selbst zu CO_2 , sondern durch Koppelung mit der Dehydrierung derselben oder wahrscheinlicher von irgend welcher aus derselben abgeleiteten Substanz bewerkstelligt. Ob und welche Substanzen als H-Donatoren für Nitratreduktion in Betracht kommen könnten, soll später besprochen werden.

In einem früheren Kapitel haben wir gesehen, dass der Quotient:

$$\frac{(\text{Prozentsatz des Umsatzes zur Atmung})}{(\text{Prozentsatz des Umsatzes zum Wachstum})}$$
 bei Zugabe von Nitrat als N-

Quelle je nach den C-Quellen grösser oder praktisch gleich jenem bei Zugabe von NH_3 ausfällt. Dies hängt wohl davon ab, ob der Prozess der Nitratreduktion mit oder ohne zusätzliche Späneatmung und Arbeitsleistung ausgeführt werden kann, d.i. ob und mit welcher materiellen und energetischen Ökonomie die Nitratreduktion durch Eingreifen in den Wachstumsvorgang bewirkt wird. In diesem Sinne scheint also der Prozess der Nitratreduktion bei verschiedenen Zuckerarten und mehrwertigen Alkoholen "günstiger" vollzogen zu werden als z.B. bei Gluconsäure und Äthylalkohol.

VI. Die alkoholische Gärung und der Pasteur-Effekt

Früher wurde von einigen Forschern (Korschelt (36); Juhler (32); Jörgensen (31); Hansen (28)) angenommen, dass die alkoholische Gärung bei der Sakebrauerei einzig und allein durch *Aspergillus oryzae* selbst bewirkt werde, indem dessen Konidien, der Meinung dieser Autoren zufolge, unter gewissen Bedingungen sich in gärfähige Hefezellen umwandeln könnten. Dass aber die in der Sakemaische entdeckte Hefe und *Aspergillus* wesentlich verschiedene Lebewesen sind, wurde von Y. Kozai und K. Yabe (49) sichergestellt. Seitdem hat man lange nicht daran gezweifelt, dass die Rolle von *Aspergillus* in der Sakebrauerei nur in der diastatischen Spaltung von Reisstärke, nicht aber in der Alkoholbildung bestehe.

Inzwischen wurde von einigen Autoren geltend gemacht, dass auch dem *Aspergillus* die Fähigkeit zukommt, bei O_2 -Abschluss eine gewisse, wenn auch ganz schwache, alkoholische Gärung zu bewirken, dass aber dabei der Pilz sehr stark an Lebensaktivität einbüsst oder sogar bald getötet wird.

(Vergl., z.B. Kostytschew (43, 44); Kostytschew und Afanassjewa (48); Diakonow (19, 20); Dude (21).) Bei *Aspergillus oryzae* hat aber Tamiya (93) dargetan, dass der Pilz durch O_2 -Entzug zwar sein Wachstum gänzlich einstellt, aber keineswegs getötet wird, indem selbst bei langdauernder Anaerobiose noch völlig die Fähigkeit beibehalten bleibt, bei erneuter O_2 -Zufuhr wieder normal zu wachsen. Ausserdem konnte er zeigen, dass die Gärfähigkeit von *Aspergillus oryzae* viel stärker ist, als sie bisher allgemein angenommen worden war, und zwar dass die Bildung von Alkohol und Kohlensäure aus Kohlehydraten nicht nur bei gänzlichem O_2 -Abschluss allein, sondern unter Umständen auch selbst unter normaler O_2 -Zufuhr—allerdings in viel geringerem Masse als bei O_2 -Abschluss—beobachtet werden kann.* Bemerkenswert ist der Umstand, dass *Aspergillus oryzae* selbst unter normaler Luftzufuhr zu starker Alkoholgärung veranlasst wird, wenn dessen Luftmyzelien mit der Nährlösung benetzt oder in die Nährlösung eingetaucht werden. Allerdings ist die Gärfähigkeit von *Aspergillus oryzae* im Vergleich mit derjenigen von Brauereihefen bedeutend schwächer, indem der Q_{CO_2} -Wert (die bei O_2 -Abschluss von 1 gm. Pilz in 1 Std. produzierte Menge der Gärungskohlensäure in cc.) höchstens ca. 10—gegenüber 200–300 bei Hefen—, und das Verhältnis:

$\frac{\text{(Gärungskohlensäure bei } O_2\text{-Abschluss)}}{\text{(Atmungskohlensäure bei } O_2\text{-Zufuhr)}}$ ungefähr 0.25–0.3—gegenüber

3–25 bei Hefen (Warburg (123))—beträgt. Je nach den *Aspergillus* Arten variiert das Gärvermögen sehr weitgehend: von den 21 untersuchten Arten erwies sich *Asp. clavatus* als ein besonders kräftiger Gärungserreger, indem dessen Gärfähigkeit ca. 10 mal grösser als diejenige von *Asp. oryzae* ist. *Aspergillus gymnosardae* und *Asp. niger* vergären beinahe ebenso stark wie *Asp. oryzae*, während *Asp. giganteus*, *fumigatus*, *nidulans* und *glauca* nur sehr wenig oder gar nicht gärfähig waren (Tamiya und Miwa (106)).†

Aus *Asp. clavatus*, *gymnosardae*, *oryzae* und *niger* lässt sich durch Mazerieren mit 50–75% Glycerinlösung ein Zymase-Komplex extrahieren, der allem Anscheine nach mit demjenigen von Hefen identisch ist (Tamiya und

* Wegen des Einsetzens dieser "aeroben Gärung" fällt der RQ -Wert bei Zugabe von gut vergärbaren Zuckern stets etwas grösser aus als der theoretisch nach den früher angeführten Bilanzgleichungen berechnete Wert. (Vergl. Tabelle III.)

† Selbst bei einer bestimmten Pilzart ist die Gärfähigkeit oft je nach den Stämmen veränderlich; in vergleichenden Gärproben an mehr als 70 Stämmen von *Aspergillus oryzae* fand K. Sakaguchi (80) einige Stämme, deren Gärfähigkeit fast derjenigen von Sake-Hefen gleichkommt.

Miwa (106)).* Bei der Gärung von intakten Zellen von *Aspergillus oryzae* wurde jedoch beobachtet, dass neben Zymohexosen auch Galactose sowie verschiedene Polysaccharide (Dextrin, Stärke, Inulin und Glykogen), die von Hefen gar nicht oder erst nach "Gewöhnung" angegriffen werden, recht kräftig vergoren werden, und dass andererseits Dioxyaceton, das von Hefen ziemlich gut vergoren wird, durch *Aspergillus* sogut wie gar nicht angegriffen wird (Tamiya (98)). Die Vergärbarkeit von Polysacchariden ist ohne weiteres durch den hohen Gehalt des Pilzkörpers an polysaccharidspaltenden Enzymen zu erklären. Nach R. Willstätter und H. Sobotka (127) wird Galactose bei Hefen durch einen besonderen Enzymkomplex (Galactozymase), der von der echten Zymase verschieden ist, vergoren. Ob man auch bei *Aspergillus* einen solchen besonderen Zymasekomplex für

TABELLE XIII

EINFLUSS DES O₂-DRUCKS AUF ATMUNG, GÄRUNG, WACHSTUM UND DEN OXYDATIONS-
QUOTIENTEN

(VERSUCH AN EINER DECKENKULTUR VON *Aspergillus oryzae*: VERSUCHSDAUER: 22.5
STDN.)

Dargereichte Gas Mischung (%)	N ₂ O ₂	100 0	83 17	67 33	50 50	33 67	17 83
Wachstums- grösse	(μ)	-0.006	0.014	0.018	0.022	0.024	0.026
Atmungs- grösse	(Q _{O₂})	0	9.8	15.3	21.7	31.7	34.1
Gärungs- grösse	(Q _{CO₂})	$Q_{CO_2}^{N_2} = 9.3$	8.6	8.4	5.9	2.0	0.7
Oxydations- quotient	(OQ)	...	0.2	0.2	0.5	0.7	0.8

Galactose anzunehmen hat, bleibt zur Zeit noch offen. Ebenso bedarf die Ursache der Unangreifbarkeit von Dioxyaceton bei *Aspergillus* noch einer näheren Erforschung.

Früher hat S. Kostytschew (41, 42, 45, 46, 47) angegeben, dass verschiedene organische Verbindungen (wie Chinasäure, Milchsäure, Weinsäure,

* Entsprechend der typischen Gärungsgleichung beträgt das Verhältnis CO₂:Alkohol bei *Aspergillus*-Gärung 100:96.3 (Tamiya (93)). Auch die Anwesenheit von Co-Carboxylase (Thiamin-Pyrophosphat) sowie von Thiamin (Vitamin B₁) im *Aspergillus*-Körper ist von mehreren Autoren nachgewiesen worden (Vergl. z.B. Nagayama (67), Takata (91, 92), Kawamura (33), Gorrica u.a. (26)).

Mannit, Glycerin u.a.), die im normalen Pilzstoffwechsel gut verwertbar sind, bei O_2 -Abschluss, analog den Zymohexosen, in Alkohol und Kohlensäure gespalten werden. Nach unserer Erfahrung entspricht diese Angabe nicht der Wirklichkeit; alle mehrwertigen Alkoholesowie organischen Säuren —ausgenommen nur Brenztraubensäure, die carboxylatisch gespalten wird, —werden bei O_2 -Abschluss von *Aspergillus oryzae* gar nicht vergoren (Tamiya (98)).

Bei normaler aerober Kultur des Pilzes auf Kohlehydraten wird die alkoholische Gärung deutlich herabgedrückt oder, bei minder gut vergärbaren Zymohexosen, gänzlich sistiert. Es findet nämlich ein deutlicher Pasteur-Effekt statt. Wie schon erwähnt, ist die aerobe Atmung von *Aspergillus* sehr empfindlich gegenüber der Schwankung des O_2 -Drucks; die stärkste Atmung findet in einer Atmosphäre von etwa 85% O_2 + 15% N_2 statt, und schon bei Herabsetzung der O_2 -Menge auf 30% kommt eine Abschwächung der Atmung zu stande. Tabelle XIII zeigt, wie die "aerobe Gärung" ($Q_{CO_2}^{O_2}$: die unter O_2 -Zufuhr von 1 gm. Pilz in einer Stunde produzierte Gärungskohlensäure) in steigendem Masse mit der Verminderung des Q_{O_2} -Wertes bei vermindertem O_2 -Druck eintritt.*

Aus der Atmungsgrösse, der Gärungsgrösse beim O_2 -Abschluss und der Grösse der aeroben Gärung lässt sich der sogen. Oxydationsquotient ausrechnen†, der, wie aus der Tabelle ersichtlich, um so grösser ausfällt je grösser die dargereicherte O_2 -Menge ist. Wie diese Erscheinung zu deuten sei, bleibt jetzt noch unklar. Hingewiesen sei darauf, dass der betreffende

* Die Grösse der aeroben Gärung $Q_{CO_2}^{O_2}$ wurde dadurch ermittelt, dass man von der Gesamtmenge der produzierten Kohlensäure die Menge der Atmungs- CO_2 (bei Kohlehydraten gleich der Menge des Atmungs- O_2) und die der Wachstums- CO_2 (berechnet nach der Wachstumsgrösse unter Berücksichtigung der Bilanzgleichung (3)) in Abzug bringt.

† Der Oxydationsquotient (OQ), d.h.
$$\frac{\text{(Von der Gärung bewahrte Zuckermoleküle)}}{\text{(Veratmete Zuckermoleküle)}}$$
 wurde aus folgender Formel ausgerechnet:

$$OQ = \frac{Q_{CO_2}^{N_2} - Q_{CO_2}^{O_2}}{Q_{O_2}} \times 3$$

worin $Q_{CO_2}^{N_2}$ = die Gärungsintensität bei vollständigem O_2 -Abschluss, und $Q_{CO_2}^{O_2}$ = die aerobe Gärung bedeutet. Der Multiplikator 3 kommt deshalb in Betracht, weil 1 cc. Gärungs- CO_2 4 mg. vergärem Zucker, und 1 cc. Atmungs- O_2 1.33 mg. veratmetem Zucker entspricht.

Wert (0.2–0.8) bei *Aspergillus* viel kleiner ist, als es bei Hefe oder Muskel der Fall ist (3–6) (Vergl. z.B. Meyerhof (53, 54)).*

Bemerkenswert ist noch die Tatsache, dass bei Zugabe von Kohlenoxyd eine deutliche Verkleinerung des Oxydationsquotienten zustande kommt (Tamiya (95)). (Vergl. Tabelle XIV.) Diese im Jahre 1929 bei *Aspergillus* konstatierte Tatsache wurde später auch bei tierischen Geweben (Retina, Allantois und Chorion) von H. Laser (52) bestätigt gefunden, der ausserdem die interessante Beobachtung machte, dass die CO-Hemmung des Pasteur-Effektes, analog der CO-Hemmung der Atmung, durch Licht teilweise aufgehoben werden kann. Ausgehend von dieser Tatsache, glaubten kürzlich K. G. Stern, J. L. Melnick und D. DuBois (84, 85) bei Anwendung der photochemischen Technik von O. Warburg die Behauptung aufstellen zu sollen, dass bei dem Pasteur-Effekt ein Eisenporphyrin-Protein mit den CO-Banden bei 570–590 (α), 500–520 (β) und 440–455 (γ) $m\mu$ beteiligt ist.

TABELLE XIV

EINFLUSS DES KOHLENOXYDS AUF DIE ATMUNG, DAS WACHSTUM UND DEN PASTEUR-EFFEKT

(Deckenkultur von *Aspergillus oryzae*: Versuchsdauer: 23 Stdn.)

Dargereichte Gasmischung (%)	N ₂ O ₂ CO	100 0 0	50 50 0	40 50 10	30 50 20	20 50 30	10 50 40	0 50 50
Wachstumsgrösse	(μ)	...	0.038	0.028	0.023	0.018	0.015	0.016
Atmungsgrösse	(Q_{O_2})	0	31.7	36.8	40.1	40.3	41.9	40.1
Gärungsgrösse	($Q_{C_2H_5O_2}$)	$Q_{CO_2}^{N_2} = 10.8$	5.6	10.0	9.7	7.7	10.2	10.4
Oxydationsquotient	(OQ)	...	0.5	0.1	0.1	0.2	0.0	0.0

VII. Das Eisenkatalysatoren-System

Aus der vorstehenden Tabelle entnimmt man, dass die Atmung von *Aspergillus*, im Gegensatz zu derjenigen bei Hefen u.a., durch Kohlenoxyd gar nicht gehemmt, sondern vielmehr gesteigert wird. Ferner ist darauf aufmerksam zu machen, dass bei CO-Zugabe immer eine unverkennbare Wachstumshemmung eintritt. Diese CO-Empfindlichkeit des Wachstumsvorgangs ist so auffallend, dass eine Verzögerung der Pilzgewichtszunahme schon bei Zugabe von 10% CO zustande kommt (Tamiya (95, 97);

* Bezüglich der physiologischen Bedeutung des Pasteur Effektes, der heute nicht mehr im Sinne der Theorie Meyerhofs gedeutet werden kann, möchte ich auf die Darlegung von D. Burk (9) verweisen.

vergl. auch Yamamoto (132)). Merkwürdigerweise ist die CO-Hemmung des Wachstums weder photochemisch aufhebbar,* noch durch nachträgliche Zugabe der CO-freien und O₂-reichen Gasmischung auszugleichen, indem der Pilz, der z.B. in einer Gasmischung 90% CO + 10% O₂ 4 Std. lang stehen gelassen war, bei nachträglicher Überführung in eine Gasmischung bestehend aus 85% O₂ + 15% N₂ noch immer um 80% an seiner Wachstumsfähigkeit beeinträchtigt blieb (Tamiya (97)). Diese Beobachtung gab dem Verfasser Anlass, darauf zu schliessen, dass bei *Aspergillus* die etwaige scheinbare Herabsetzung der O₂-Aufnahme, die bei Zugabe einer grösseren CO-Menge (z.B. 90%) eintreten kann, nicht, wie es O. Warburg in seiner Arbeit über das O₂-übertragende Ferment annimmt, im Sinne der Blockierung des Atmungsenzymys durch CO, sondern vielmehr als Folge der Verminderung der Zahl der atmenden Zellen zu deuten sei. Immerhin lag dabei der Schluss nahe, dass die Warburgsche Theorie des Atmungsfermentes nicht auf die Fälle von *Aspergillus* übertragen werden kann.† Der Schlussfolgerung von Tamiya wurde aber von Warburg und Kubowitz (125) mit dem Hinweis begegnet, dass die Atmung von *Aspergillus oryzae* bei ihrem manometrischen Versuch tatsächlich eine reversible 26%-ige CO-Hemmung (in einer Gasmischung aus 95% CO + 5% O₂) erlitt, dass also die Atmung von diesem Pilz keineswegs eine Ausnahme von der Warburgschen Theorie bilden könnte.

Weitere Untersuchungen in unserem Laboratorium haben zu der Auffassung geführt, dass sich zwar sowohl unsere wie auch die Warburgschen Beobachtungen an sich völlig bestätigen—indem die widersprechenden Ergebnisse in den beiden Fällen nur auf der Verschiedenheit der Versuchsbedingungen beruhen —, dass aber die von Warburg u.a. beobachtete CO-Hemmung immer noch kaum als eine normale Erscheinung betrachtet werden darf. Der Grund dafür sei unten etwas näher dargelegt, weil man dadurch weitere Einsicht in die charakteristischen Eigenschaften des Pilzstoffwechsels, die in mancher Hinsicht denjenigen der meisten Organismen schlagend gegenüberstehen, gewinnen kann.

Wie schon erwähnt, kann man durch die Durchlüftungskultur sehr feine submerse Hyphenfitzchen erhalten, die gleichmässig in der Kulturlösung suspendiert sind. Merkwürdigerweise wird die Atmung von diesen sub-

* Unveröffentlichte Arbeit von K. Ohta.

† Unser *Aspergillus* war der erste, der sich als ein Organismus erwies, der die seinerzeit von O. Warburg postulierte CO-empfindliche Atmung nicht zeigt. Kurz darnach wurde aber auch von mehreren anderen Autoren die CO-unempfindliche, ja sogar durch CO gesteigerte Atmung bei verschiedenen Objekten beobachtet (vergl. z.B. Fenn und Cobb (23)).

mersen Hyphen, im Gegensatz zu derjenigen von Deckenpilz, ziemlich deutlich durch CO gehemmt, und übrigens wird diese Hemmung, wie bei Hefen, durch Licht teilweise beseitigt (Ogura und Nagahisa (71)). (Vergl. Tabelle XV.)

TABELLE XV

EINFLUSS DES KOHLENOXYDS AUF DIE ATMUNG VON SUBMERSEN HYPHEN VON *Aspergillus oryzae*

Dargereichte Gas Mischung	95% N ₂ + 5% O ₂	95% CO + 5% O ₂	
		Dunkel	Hell
Q _{O₂}	48.5	26.5	30.2
Hemmungs, %	45.4	37.8

Bemerkenswert ist dabei noch, dass die Atmung der submersen Hyphen gegenüber Blausäure viel empfindlicher ist als die des gewöhnlichen Deckenpilzes. In Tabelle XVI sind die Ergebnisse der von Tamiya (95) an Deckenpilz und der von Ogura und Nagahisa (71) an submersen Hyphen von demselben Pilzstamm ausgeführten Versuche zusammengestellt. So wird die Atmung von submersen Hyphen durch M/1000 CN um etwa 80% gehemmt, während diejenige von Deckenpilz dadurch nur noch 14% Hemmung erleidet; 80%-ige Hemmung kommt in diesem letzteren Fall auch bei Zugabe von M/100 Blausäure noch nicht zustande. Dass der Pilz *Aspergillus* unter normalen Kulturbedingungen sehr widerstandsfähig gegen Blausäure ist, und dass er diese Substanz sogar als N-Quelle auszunutzen vermag, ist eine schon lange bekannte Tatsache (vergl. z.B. Ohtsuki (72)).

Die unterschiedliche CO- und CN-Empfindlichkeit von submersen Hyphen und von Deckenpilzen deutet darauf hin, dass bei den ersteren die Atmung wohl hauptsächlich durch das Eisenkatalysatorsystem vermittelt wird, während die Rolle desselben bei der Atmung von Deckenpilzen, wenn nicht gänzlich, so doch zum grössten Teil von irgend einem CO- und CN-unempfindlichen, höchstwahrscheinlich nicht eisenhaltigen Katalysator übernommen ist. Dass aber die Atmung von submersen Hyphen durch CN nicht gänzlich sistiert wird, muss so gedeutet werden, dass darin neben dem Eisenkatalysatorsystem noch eine geringe Menge von nicht-eisenhaltigem O₂-Übertragungssystem enthalten sei (vergl. hierzu Tamiya und Kubo (105)). Ebenso liegt es nahe anzunehmen, dass im Deckenpilz neben einer grösseren Menge von einem eisenfreien Enzymsystem, eine geringere Menge von Eisenkatalysatorsystem vorhanden sei. Die Verhältnisse lassen sich klar und eindeutig daraus ersehen, dass das Cytochrom und die

Cytochromoxydase ("Nadi"-Oxydase) in Deckenpilzen zwar nicht gänzlich fehlen, aber nur in sehr kleinen Mengen enthalten sind, während sie in submersen Hyphen in bedeutend grösserer Mengen nachweisbar sind. K. Shibata und H. Tamiya (82) haben dargetan, dass der Cytochromgehalt in der Decke von *Aspergillus oryzae* (5-tägige Kultur), obwohl dessen Atmungsintensität derjenigen der Bäckerhefe nicht nachsteht, weniger als $\frac{1}{10}$ von derjenigen bei Hefezellen ist. Übrigens nimmt der Cytochrom- und Cytochromoxydase-Gehalt mit dem Fortschreiten der Kultur sehr schnell ab, indem bei 12-tägigen Decken, die noch recht intensiv atmeten ($Q_{O_2} = 21$), weder Cytochrombanden noch die "Nadi"-Reaktion mehr

TABELLE XVI

EINFLUSS DER BLAUSÄURE AUF DIE ATMUNG VON PILZDECKEN UND SUBMERSEN HYPHEN VON *Aspergillus oryzae*

	Kontrolle	CN-Zugabe		
		M/1000	M/500	M/100
Deckenpilz				
Q_{O_2}	41.1	35.5	33.9	11.8
Hemmungs, %	...	14	18	71
Submerse Hyphen				
Q_{O_2}	72.6	15.9	11.0	...
Hemmungs, %	...	78	85	...

wahrzunehmen waren.* Dagegen zeigen die submersen Hyphen, deren Atmungsintensität nicht besonders grösser als die von Pilzdecken ist, eine viel stärkere "Nadi"-Reaktion sowie etwa 5–10 mal mehr Cytochromgehalt—also beinahe denselben wie bei Hefe—als die ganz junge Pilzdecke (Ogura und Nagahisa (71)).

In einem früheren Kapitel wurde darauf hingewiesen, dass die Atmung des Deckenpilzes gegenüber der Schwankung des O_2 -Drucks in der Atmosphäre bedeutend empfindlicher ist als diejenige von submersen Hyphen. Dieser Tatbestand ist dadurch erklärbar, dass der in Frage kommende

* In jüngeren Decken scheint übrigens das Cytochrom nur in unterer Schicht der Decke lokalisiert vorzukommen, weil auf die Benzidinprobe nur dieser Teil positive Reaktion aufweist (Ogura und Nagahisa (71)). Tamiya (94) hat beobachtet, dass sowohl die Cytochrombanden wie auch die "Nadi"-Reaktion in jüngeren Pilzdecken bei O_2 -Entzug sehr bald (innerhalb etwa 5 Stdn.) verloren gehen. Bei Behandlung solcher Decken mit Pyridin sind die starken Banden des Pyridinhämochromogens zu beobachten. Vielleicht wird das Cytochrom durch Anaerobiose nicht zerlegt, sondern in Parahämatin verwandelt.

nicht CO- und CN-empfindliche Atmungskatalysator eine viel kleinere Affinität zum Sauerstoff besitzt als die Eisenkatalysatorsysteme.

Bezeichnen wir mit

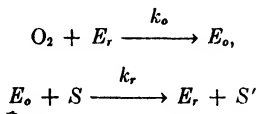
E_o und E_r = die oxydierte bzw. reduzierte Form des Enzyms oder des Enzymsystems überhaupt, welches zwischen Sauerstoff und Atmungssubstrat eingeschaltet die Reaktion von diesen beiden katalysiert,

S und S' = das Atmungssubstrat bzw. dessen Oxydationsprodukt,

k_o = die Geschwindigkeitskonstante bei der Reaktion zwischen Sauerstoff und E_r ,

k_r = die Geschwindigkeitskonstante bei der Reaktion zwischen E_o und S ,

so ist der Mechanismus der Atmung in grossen Zügen wie folgt zu formulieren:



Es lautet:

$$\frac{-dE_r}{dt} = k_o[\text{O}_2][E_r] - k_r[E_o][S]$$

Bei stationärem Zustand ist $(-dE_r/dt) = 0$, und folglich ist

$$\begin{aligned} \frac{[E_r]}{[E_o]} &= \frac{k_r[S]}{k_o[\text{O}_2]}, \\ \frac{[E_r]}{[E_o] + [E_r]} &= \frac{k_r[S]}{k_r[S] + k_o[\text{O}_2]} \end{aligned}$$

Es sei ferner

v = die Geschwindigkeit der O_2 -Aufnahme, die durch das betreffende Enzymsystem katalysiert wird,

$[E]$ = die Gesamtkonzentration des betreffenden Enzymsystems, d.i. $[E_o] + [E_r]$,

dann ist

$$v = k_o[\text{O}_2][E_r],$$

oder

$$v = \frac{k_o k_r [\text{O}_2][S][E]}{k_r[S] + k_o[\text{O}_2]} \quad (15)$$

$[\text{O}_2]$ stellt die Konzentration des Sauerstoffs an der Enzymoberfläche dar; bezeichnet man dies mit p , und ferner sei

P = die Sauerstoffkonzentration in der Atmosphäre,

a = der Abstand zwischen der Atmosphäre und der Enzymoberfläche,

D = die Diffusionskonstante des Sauerstoffs in dem Medium zwischen Atmosphäre und Enzymoberfläche,

F = der Flächeninhalt des Mediums, durch welchen Sauerstoff diffundiert,

so ist nach dem Fickschen Diffusionsgesetz:

$$v = \frac{DF}{a} (P - p) \quad (16)$$

Die Formel (15) besagt, dass bei Konstanz von $k_r[S]$ und $[E]$ die funktionelle Beziehung zwischen v und p (d.i. $[O_2]$) eine Kurve in Form einer rechtwinkligen Hyperbel darstellt. Ist k_o genügend gross, so ist (insofern als p nicht allzu klein und $k_r[S]$ nicht allzu gross ist)

$$v = k_r[S][E]. \quad (17)$$

Ist dagegen k_o sehr klein, sodass $k_o[O_2]$ gegenüber $k_r[S]$ vernachlässigbar sein könnte, so ergibt sich aus (15)

$$v = k_o p [E] \quad (18)$$

Aus (16) und (18) lässt sich ableiten:

$$v = \frac{k_o DF [E] P}{a k_o [E] + DF} \quad (19)$$

Nun dürften in *Aspergillus* zwei Enzyme (oder besser Enzymsysteme) enthalten sein, wovon das eine, nämlich CN- und CO-empfindlicher Eisenkatalysator, einen sehr grossen k_o -Wert besitzt, während dem anderen, einem Nicht-Eisenkatalysator, ein kleinerer k_o -Wert zukommt. Bezeichnen wir das erste Enzym mit E_1 und das andere mit E_2 , und die Geschwindigkeit der dadurch bewerkstelligten O_2 -Aufnahme mit v_1 bzw. v_2 , so ist die Atmungsgeschwindigkeit V von *Aspergillus*—unter der oben erwähnten Bedingung—wie folgt auszudrücken, indem bei submersen Hyphen $[E_1] > [E_2]$, bei Deckenpilzen hingegen $[E_1] < [E_2]$ sein sollte.

$$\begin{aligned} V &= v_1 + v_2 \\ &= k_{r1}[S][E_1] + \frac{k_{o2}DF[E_2]P}{a k_{o2}[E_2] + DF} \end{aligned} \quad (20)$$

Wegen des Umstandes, dass bei submersen Hyphen $E_1 > E_2$, und a recht gross ist, weil sie im Innern der Versuchsflüssigkeit versenkt sind, wird das zweite Glied der rechten Seite der Gleichung (20) gegenüber dem ersten Glied bedeutend kleiner, sodass die Atmung von submersen Hyphen, wie die Versuche von Ogura und Nagahisa zeigen, sich gegenüber der Schwankung von P weitgehend unabhängig und gegen CO und CN ziemlich empfindlich zeigt. Bei dem Deckenpilz ist die Sachlage umgekehrt; wegen $[E_1] < [E_2]$ und kleineren a -Wertes—weil die atmenden Hyphen sich in der Luft befinden—ist das Verhältnis von v_1 zu v_2 bedeutend zugunsten des letzteren vergrössert, wodurch wohl erklärbar ist, warum die normale Atmung des Deckenpilzes gegenüber CN und CO refraktär, aber sehr von der Grösse von P abhängig ist. Wird der Deckenpilz ins Innere der Kulturlösung versenkt, so wird wegen der Vergrösserung von a der Wert von v_2

verringert, wodurch zu verstehen ist, warum der Deckenpilz beim Eintauchen in die Lösung eine starke Gärung bewirkt.

Wir sind jetzt imstande, die Ursache der von Warburg beobachteten CO-Hemmung der *Aspergillus*-Atmung befriedigend zu erklären. Im Gegensatz zu unserer Versuchsanordnung, in welcher stets dafür gesorgt wurde, dass der Deckenpilz normalerweise unter freier Sauerstoffzufuhr atmen kann, wurde bei Warburgschen Versuchen die Atmung des *Deckenpilzes* unter Eintauchen desselben in Versuchsflüssigkeit manometrisch gemessen. Aus den erwähnten Gründen musste dabei v_2 bedeutend in den Hintergrund getreten sein, und anstatt dessen muss sich die CO-empfindliche Atmung v_1 , wenn auch deren absolute Grösse sehr klein ist, doch bemerkbar gemacht haben. Dass die unter derartigen Bedingungen beobachtete CO-Hemmung kaum als eine normale Eigenschaft der *Aspergillus*-Atmung angesprochen werden kann, versteht sich wohl von selbst.

Nach alledem glauben wir schliessen zu dürfen, dass bei der Atmung von *Aspergillus* ein besonderes Redoxsystem, das allem Anscheine nach nicht von Häminnatur ist, die hauptsächlich Rolle eines O_2 -übertragenden Enzyms spielt. Über die chemische Natur dieses Enzyms kann zur Zeit nichts ausgesagt werden. Nicht ausgeschlossen ist die Möglichkeit, dass es ein Flavin ist, dessen Anwesenheit in Myzelien von *Aspergillus* schon von mehreren Autoren bestätigt gefunden ist (Vergl. z.B. Scheunert und Schieblisch (81)). In diesem Zusammenhang ist die Angabe von W. Franke und M. Deffner (24) erwähnenswert, dass die Glucosedehydrase von *Aspergillus*, worüber in dem nächsten Kapitel eingehend gesprochen werden soll, höchstwahrscheinlich eine Art Flavinenzym ist.

Hier finde ich es am Platze, ausgehend von den oben dargelegten Betrachtungen eine Bemerkung darüber zu machen, dass in der Natur überhaupt zwei Typen der aeroben Lebewesen existieren, die sich nach ihrer Atmungs- und Lebensweise voneinander unterscheiden lassen. Damit in der Natur überhaupt die aeroben Organismen die Atmung:



mit einer bestimmten Geschwindigkeit sowie mit einem bestimmten Energiegewinn ausführen können, muss nach dem Massenwirkungsgesetz entweder die Konzentration des Sauerstoffs oder die des Substrates entsprechend gesteigert werden; das günstigste Verhältnis besteht natürlich in der gleichzeitigen Erhöhung dieser beiden Konzentrationen, was aber in der Natur nur selten realisierbar ist. Bei einer solchen Lage hat der Organismus die Wahl zu treffen, entweder unter Verzicht auf die grössere O_2 -Konzentration den Ort höherer Substratkonzentration (S) zu suchen oder umgekehrt. Um bei niedrigerer O_2 -Konzentration und grösserer Substratmenge starke Atmung auszuführen, muss der Organismus, wie aus der Formel (15) ersichtlich, eine grössere Menge von jenem Enzymsystem besitzen, welchem ein grösserer k_s -Wert zukommt. Diesem

Zweck entspricht gerade das Eisenkatalysatorsystem, dessen Affinität zu Sauerstoff, wie Warburg und Kubowitz (124) nachgewiesen haben, beträchtlich ist. Den Organismus, der mit Hilfe eines solchen Enzymapparates seine aeroben Lebensleistungen ausübt, finden wir bei allen submers vegetierenden Aerobiern, wie Hefen, Bakterien u.a., und ferner ganz allgemein bei Tieren, deren Körperorganisation, wie die entwicklungs-geschichtlichen Betrachtungen uns lehren, in ihrem allgemeinen Bestreben nach dem "Intensivwerden" charakterisiert ist. Dies hat notwendigerweise eine immer schwierigere O_2 -Zufuhr ins Körperinnere zur Folge. Diese Sachlage ist beim Tiere gesichert durch auffallende Entwicklung des Eisenkatalysatorensystems, und zwar kommen dabei als solches nicht nur verschiedene intrazelluläre Eisensysteme, wie Cytochrome und O_2 -übertragendes Ferment Warburgs, sondern auch als deren Ergänzung noch Muskelhämoglobin sowie Bluthämoglobin zum Vorschein. Andererseits kann die Beweglichkeit des Körpers, die als ein wesentliches Charakteristikum des Tieres zu betrachten ist, als zweckdienlich für seine Lebensweise, den Ort der höheren Substratkonzentration zu suchen, gedeutet werden.

Nun gibt es in der Natur eine andere Gruppe der aeroben Organismen, die eine bessere O_2 -Zufuhr begehren unter Verzicht auf die höhere Substratkonzentration. Um diesen Bedarf zu decken, haben sie nach Möglichkeit eine grosse Körperoberfläche (F) und nach Möglichkeit einen kurzen Diffusionsweg des Sauerstoffs (a), nämlich sie haben "extensive" Körperorganisation und atmen mit den Zellen, die unmittelbar mit der Luft in Berührung stehen. Wie aus der Formel (19) ersichtlich, können die Organismen mit solcher Körperorganisation ein Enzymsystem mit grösseren k_s -Wert entbehren, sie können also ohne Eisenkatalysatorsystem genügend starke Atmung bewirken. Zu dieser Organismengruppe gehören Schimmelpilze sowie andere in freier Luft ausgesetzt vegetierende Organismen, deren höchst entwickelte Form wir in höheren Pflanzen finden. Charakteristisch für solche Organismen ist die Anspruchslosigkeit gegenüber dem Atmungssubstrat sowie ferner die Unbeweglichkeit des Körpers. Es sei nur daran erinnert, dass die Schimmelpilze oft auf so nährstoffarmen Orten vegetieren, wie auf der Oberfläche der Linsen der Kamera oder des Fernrohrs, wo als C-Quelle nur die Verunreinigungen aus dem Luftstaub in Betracht kommen können. Derartige Anspruchslosigkeit finden wir freilich nie bei Tieren und auch kaum bei submers lebenden Mikroorganismen wie Hefen und Bakterien.

Wir glauben heute schon ziemlich viel über den Mechanismus der Atmung von denjenigen Organismen kennengelernt zu haben, die submerses Leben treiben oder "intensive" Körperorganisation besitzen. Hoffentlich gelingt es der Zukunft, auch eine ebenso tiefgreifende Erforschung über den Atmungsmechanismus der anderen Organismengruppe durchzuführen, eine Arbeit, die bisher in der Physiologie sowie der Enzymologie fast ganz brach liegt.

VIII. Die Dehydrasen

Abgesehen von der wohl bekannten Untersuchung von D. Müller (56, 57, 58, 59, 60, 63) über die Glucosedehydrase von *Aspergillus niger*—die allerdings von Müller selbst als eine Oxydase angesprochen worden ist—gibt es in der Literatur nur selten Arbeiten, die sich mit der Frage der Dehydrasen in Schimmelpilzzellen befassen. Im Jahre 1929 haben Tamiya und Hida (103) die Anwesenheit von Succinodehydrase in *Aspergillus*

oryzae nachgewiesen. In seinem Präparat aus *Aspergillus niger* fand Müller (60) eine Dehydrase, die auf Äpfelsäure dehydrierend wirkte. Sonst lagen in früheren Zeiten—soweit mir bekannt—keine Angabe über die Dehydrase in *Aspergillus* vor.

Im Jahre 1929, als ich (96) zum erstenmal an *Aspergillus oryzae* eine Untersuchung mit Thunbergscher Technik anstellte, beobachtete ich eine merkwürdige Tatsache, dass nämlich die Kulturlösung, die von der Pilzdecke abgetrennt wurde, an und für sich die Fähigkeit entfaltete, unter Luftabschluss Methylenblau zu entfärben. Aus dieser Tatsache habe ich seinerzeit darauf geschlossen, dass der Pilz eine gewisse Dehydrase in die Kulturlösung ausscheidet, die auf Kosten von irgend einem in der Nährlösung befindlichen Substrat Methylenblau reduziert. Sonderbarerweise findet diese Methylenblaureduktion nur dann statt, wenn das Thunberg-Rohr an einen hellen Ort gestellt wird, und wenn man den Versuchsansatz, in welchem unter Belichtung die Methylenblaureduktion stattgefunden hat, vor Licht schützt, so tritt die Rückoxydation des Leukomethylenblaus ein, bis schliesslich die ursprüngliche Blaufarbe wieder in vollem Masse hergestellt wird (Tamiya, Hida und Tanaka (104)). Die Ursache dieser recht interessanten umkehrbaren Methylenblaureduktion wurde von uns näher untersucht, wobei es sich herausstellte, dass diese Erscheinung nicht, wie ich zunächst vermutete, durch die Wirkung der Pilzdehydrase, sondern durch Kojisäure, die von dem Pilz gebildet in geringer Menge in der Lösung vorhanden war, herbeigeführt worden war. Ferner wurde dargestellt, dass es sich bei der photochemischen Beschleunigung der Methylenblaureduktion nicht um die Lichtwirkung auf Kojisäure, sondern um die photochemische Aktivierung des Methylenblaus handelt. Mit der naheliegenden Annahme, dass der Wasserstoff, der auf Methylenblau übertragen

wird, von der Atomgruppierung im Kojisäuremolekül $\begin{array}{c} \text{O} \quad \text{OH} \\ | \quad | \\ -\text{C}-\text{C}=\text{C}- \end{array}$ herrühre, haben wir weiter andere Substanzen mit ähnlichen Strukturen, nämlich Acetessigester (Enolform), Brenztraubensäure (Enolform), Phloroglucin und Resorcin auf ihren Einfluss auf Methylenblau untersucht. Erwartungsgemäss wurde bei allen diesen Substanzen mit sogen. beweglichem Wasserstoffatom—jeweils bei bestimmtem pH-Wert—eine ähnliche photochemisch ausgelöste Methylenblaureduktion beobachtet, obwohl dabei die Reversibilität der Färbung bei nachträglichem Lichtabschluss je nach den Substanzen bald vollkommen, bald aber unvollkommen war. Derartige photochemische Beschleunigung der Farbstoffentfärbung wurde, wenn auch weniger deutlich als im obigen Fall, auch bei enzymatischer Methylenblaureduktion durch Hefezellen, Essigbakterien sowie Leber-

extrakt (Acetaldehyd als Substrat) beobachtet. Wir haben deshalb darauf hingewiesen, dass man dem Einfluss des Lichtes, den man gewöhnlich bei Methylenblauversuchen kaum in Acht zu nehmen pflegt, immer und zwar besonders dann Rechnung tragen muss, wenn die Experimente unter starker Beleuchtung ausgeführt werden.

Die von Tamiya in Angriff genommene Untersuchung der Dehydrase von *Aspergillus oryzae* wurde von Ogura und Nagahisa (71) weiter systematisch ausgedehnt, wobei die Anwesenheit von folgenden Dehydrasen (Methylenblau als H-Akzeptor) mit Sicherheit nachgewiesen werden konnte. Ferner gelang es den genannten Forschern mehrere Dehydrasen aus den Pilzzellen —unter Anwendung von *M/6* Phosphatpuffer (*pH* 8.2)—zu extrahieren, und an den zellfreien Enzympräparaten die Notwendigkeit bzw. Entbehrlichkeit der Codehydrase I näher zu prüfen.

Alkoholdehydrase: Coferment notwendig; wirkt dehydrierend auf Äthyl-, Propyl- und Butylalkohol, und auch vielleicht auf Äthylenglykol und Trimethylenglykol.*

Malicodehydrase: Coferment notwendig.

Citricodehydrase: Coferment notwendig.

β -Oxybutyricodehydrase: Coferment notwendig.

Mannitdehydrase: Coferment notwendig; dieses Enzym ist bisher nur bei *Upterhefe* nachgewiesen worden (Müller (64)).

Lacticodehydrase: Coferment vielleicht nicht notwendig.

Glycericodehydrase: Coferment vielleicht nicht notwendig.

α -Glycerinphosphatdehydrase: Coferment vielleicht nicht notwendig.

Succinodehydrase: Coferment nicht notwendig.

Glycolicodehydrase: Coferment nicht notwendig, wirkt auf Glykolsäure dehydrierend.

Hexosediphosphatdehydrase.

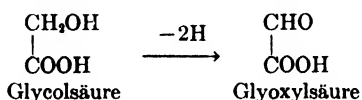
Mucicodehydrase: wirkt dehydrierend auf Schleimsäure.

Der Gehalt an solchen Enzymen war bei submersen Hyphen und bei Deckenpilzen etwas verschieden; im Grossen und Ganzen zeigten die submersen Hyphen grösseren Gehalt an coferment-bedürftigen Dehydrasen (Alkohol-, Malico-, Citrico- und Mannitdehydrase) und kleineren Gehalt an coferment-freien Dehydrasen (Glykolico-, Glycerico- und Lacticodehydrase) als der Deckenpilz. In keinem Fall wurden Formico-, Acetico-, Fumarico-, Oxalo- und Glycerodehydrase nachgewiesen. Ebenso wenig konnte die Methylenblau-reduktion bei Adonit, Erythrit, Chinasäure, Sebacinsäure, Leucin, Alanin, Glutaminsäure u.a., festgestellt werden, die ebenfalls im normalen Stoffwechsel des Pilzes gut verwertet werden.

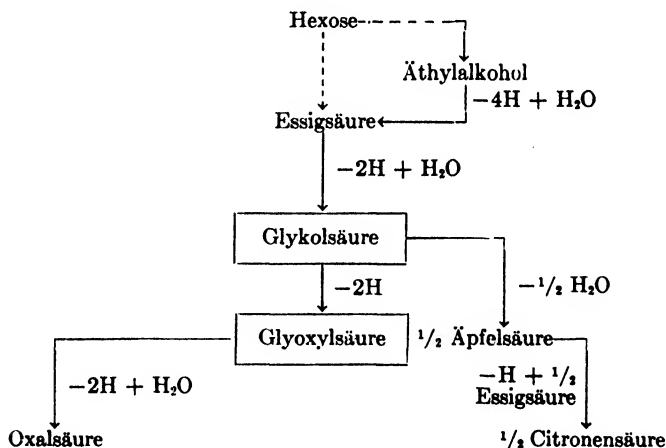
Unter den angeführten Dehydrasen sind zunächst die Glycolicodehydrase

* Vergl. hierzu den Befund von Müller bei *Unterhefe* (61, 62).

und Mucicodehydrase hervorzuheben, weil deren Anwesenheit hier zum ersten Mal nachgewiesen worden ist. Beim Experiment mit Schleimsäure wurde stets die Erscheinung beobachtet, dass das Methylenblau, nachdem einmal reduziert, allmählich wieder rücoxydiert wird. Die Ursache dieser Erscheinung sowie das Dehydrierungsprodukt der Schleimsäure ist leider noch nicht aufgeklärt. Ebenso wurde bis jetzt noch kein Versuch ausgeführt, das Dehydrierungsprodukt aus Glykolsäure, das höchstwahrscheinlich Glyoxylsäure sein dürfte, chemisch näher zu bestimmen.



Die Anwesenheit der Glycolicodehydrase in *Aspergillus* dürfte in Zusammenhang mit der wiederholt diskutierten Frage der oxydativen Gärung von Bedeutung sein. Gestützt auf die Tatsache, dass *Aspergillus niger* aus Essigsäure Glykolsäure,* und aus dieser letzteren Glyoxylsäure* sowie Oxalsäure† produziert, ist von mehreren Forschern folgendes Schema für den Mechanismus der Citronensäure-‡ und Oxalsäurebildung§ aufgestellt worden:



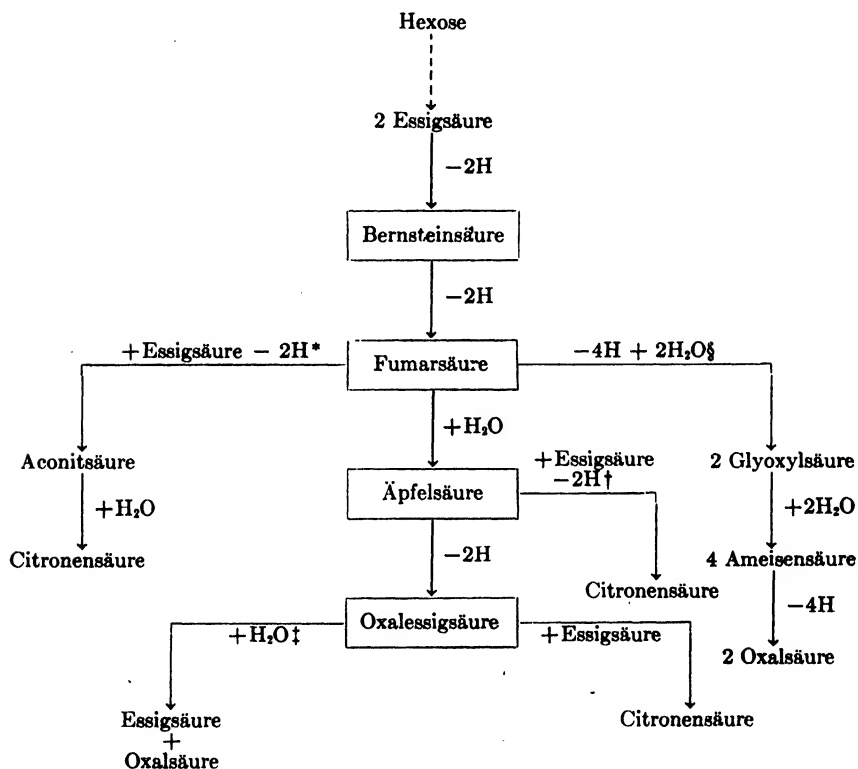
* Vergl. Challenger, Subrahmanyam, und Walker (12); sowie Bernhauer und Scheuer (5).

† Bernhauer und Slanina (7).

‡ Chrzaszcz, Tiukow und Zakomorny (13).

§ Bernhauer und Siebenäuger (6), vergl. auch Walker, Subrahmanyam und Challenger (122).

Für den Erklärungsversuch des Mechanismus der Citronensäure- und Oxalsäurebildung erscheint auch der Nachweis der Succino- und Malico-dehydrase in *Aspergillus* von Interesse, und zwar angesichts folgenden Schemas, das die bisher von mehreren Forschern vertretenen Ansichten zusammenfassend wiedergibt:



Die Anwesenheit der Fumarase in *Aspergillus* ist sehr wohl möglich, weil

* Bernhauer und Böckl (4).

† Chrzaszcz, Tiukow und Zakomorny (13), Bernhauer und Siebenäuger (6), Iwanoff und Zwetkoff (30), Gudlet, Kirsanova und Makarowa (27).

‡ Bernhauer und Siebenäuger (6).

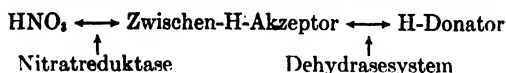
§ Chrzaszcz und Zakomorny (14).

bei *Aspergillus niger* sowohl die Bildung der Äpfelsäure aus Fumarsäure,* wie auch die Bildung der letzteren aus der ersteren,† bestätigt worden ist. Die Bildung der Äpfelsäure aus Bernsteinsäure dürfte demnach wohl rein enzymatisch möglich sein; tatsächlich ist dieser Vorgang an lebenden Pilzen von *Aspergillus niger* nachgewiesen worden (Subrahmanyam, Stent und Walker (86)). Enzymologisch bleibt in den obigen Phasenfolgen unter anderem der Vorgang der Dehydrierung von Essigsäure (zu Glykolsäure oder Bernsteinsäure) einer Klärung bedürftig, denn man konnte in *Aspergillus* gar keine Wirkung der Aceticodehydrase feststellen. Vermutlich ist hier die Sachlage ähnlich wie bei Hefen, bei welchen die Dehydrierung der Essigsäure, die bei aeroben Versuchen an intakten Zellen mit einer grossen Geschwindigkeit erfolgt, bei anaeroben Versuchen durch Farbstoffakzeptoren nur schwierig oder kaum in Gang gesetzt werden kann.

Die Frage, ob und in welchen gegenseitigen Beziehungen die oben angeführten Dehydrasen in der normalen Sauerstoffatmung ihre Funktionen entfalten, kann zur Zeit kaum beantwortet werden. Man muss natürlich die Möglichkeit in Erwägung ziehen, dass solche Dehydrasen nicht nur an der Atmung sowie Säurebildung, sondern auch an mancherlei oxydoreduktiven Vorgängen in der Zwischenstufe des Aufbaustoffwechsels beteiligt sein können. In dieser Hinsicht beansprucht die Arbeit von G. Terui einer besonderen Erwähnung, der bei *Aspergillus oryzae* eine enzymatische Oxydoreduktion zwischen Nitrat und verschiedenen organischen Substanzen nachgewiesen hat. T. Ohtsuki (72) hat festgestellt, dass der Brei oder das Acetonpräparat von *Aspergillus oryzae* eine Fähigkeit besitzt, bei Zugabe von Glucose und Nitrat dieses letztere zu Nitrit zu reduzieren. Aus Acetonpräparat von *Aspergillus oryzae* gelang es Terui (117), mit Glycerin-Phosphatpuffer ($M/10$ Phosphatpuffer von pH 5.6 mit 50% Glycerin) ein Enzymgemisch zu extrahieren, welches unter Dehydrierung verschiedener Substanzen (Glucose, Succinat, Lactat, Citrat, Tartrat u.a.) Nitrat zu Nitrit reduziert. In ihrer CN-Empfindlichkeit ähnelt die jeweilige Reaktion sehr der enzymatischen Nitratreduktion bei Bakterien, welche zuerst von I. H. Quastel, M. Stephenson und M. D. Whetham (76) untersucht, und neuerdings von S. Yamagata (130, 131) eingehend durchforscht wurde. An Hand der zellfreien Enzympräparate aus *B. coli* und *B. pyocyaneus* hat Yamagata dargetan, dass an der bakteriellen Nitratreduktion neben gewöhnlichen Dehydrasesystemen noch ein besonderes, CN-empfindliches Enzym, Nitratreduktase, beteiligt ist:

* Challenger und Klein (11).

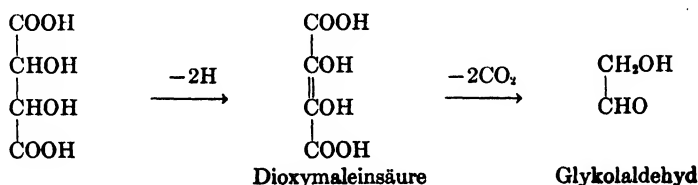
† Stent, Subrahmanyam und Walker (83).



Bei *Aspergillus*, dessen nitratreduzierende Fähigkeit viel schwächer als diejenige der genannten Bakterien ist, ist bisher noch nicht die Trennung der Nitratreduktase von den Dehydrasesystemen geglückt; jedoch liegt es durchaus nahe anzunehmen, dass auch in diesem Fall der Vorgang nach dem obigen Schema stattfindet. Es sei aber bemerkt, dass bei den Terui'schen Experimenten sich auch diejenigen Substanzen als H-Donatoren für Nitratreduktion wirksam zeigten, deren Dehydrierung bei den Versuchen von Ogura und Nagahisa nicht durch gewöhnliche Methylenblau-Methode nachgewiesen werden konnte (z.B. Fumarat, Tartrat, Malonat, Formiat, Acetat, Butyrat u.a.).* Auch die Angabe von Terui (117, 121), dass die enzymatische Nitratreduktion bei *Aspergillus oryzae*, gleichgültig welche Substanz als H-Donator verwendet wird, stets ohne Coferment erfolgen könnte, scheint schwer vereinbar mit der Annahme, dass dabei die Zusammenwirkung der gewöhnlichen Dehydrasen und eines der Nitratreduktase Yamagata's analogen Enzyms stattfindet. Ob der Nitratreduktion bei *Aspergillus* ein von jenem bei Bakterien verschiedener Mechanismus zugrunde liegt, oder ob die oben erwähnten Verhältnisse auf der Verschiedenheit der untersuchten Pilzstämme—der von Terui gebrauchte Stamm von *Aspergillus oryzae* war nicht derselbe wie derjenige von Ogura und Nagahisa—beruht, bedarf noch der Klärung.

In der oben angeführten Arbeit haben Ogura und Nagahisa die Tatsache hervorgehoben, dass bei den Methylenblau-Versuchen mit dem Pilzbrei gar keine Dehydrierung von Glucose, Mannose, Galaktose und Xylose stattfindet, dass aber merkwürdigerweise die Dehydrierung dann eintritt, wenn man anstatt des Methylenblaus Bindschedler-Grün als H-Akzeptor ver-

* Vergl. Terui (117, 118). Nach diesem Autor (119, 120) entsteht bei Dehydrierung des Succinates (Nitrat als H-Akzeptor) Tartrat und Äpfelsäure (aber kein Fumarat bzw. Oxalacetat), bei Dehydrierung von Tartrat Glykolaldehyd neben einer geringen Menge von Glyoxylsäure. Für diesen letzteren Fall ist von Terui folgende Reaktion angenommen:



wendet. Diese akzeptorspezifische Dehydrase wurde von Ogura (69) aus den Pilzzellen—unter Anwendung von $M/10$ Phosphatpuffer von pH 8.2—extrahiert, und weiter liess sie sich durch Dialyse sowie wiederholte Fällung mit Bleisubacetat, Aceton und Ammonsulfat u.s.w. als ein sehr wirksames Enzympräparat erhalten. Dieses Enzym bewirkt zwar, wie Ogura feststellen konnte, die Dehydrierung der Glucose zu Gluconsäure, ist aber, im deutlichen Gegensatz zu "Glucoseoxydase" von Müller durchaus anoxytrop, d.h. nicht direkt mit Sauerstoff reagierend. Inzwischen wurde von W. Franke und F. Lorenz (25) die bemerkenswerte Angabe gemacht, dass nämlich die Glucoseoxydase Müllers nicht, wie dieser zunächst glaubte, eine echte Oxydase, sondern eine oxytrophe Dehydrase sei, indem sie als anaeroben Akzeptor nur ~~das~~ Chinon sowie die dem Chinon—chemisch wie auch hinsichtlich der Potentialhöhe—nahestehenden Indophenole, nicht aber die Thiazin- und Oxazinfarbstoffe (wie Methylenblau und Nilblau) sowie Indogoderivate und Phenazine zu verwerten vermag.

Durch eingehende Untersuchungen von Ogura (70) einerseits und von Franke und seinen Mitarbeitern (24, 25) andererseits wurde nun eine Anzahl Tatsachen zu Tage gefördert, die eine auffallende Ähnlichkeit und sehr nahe Verwandtschaft zwischen den beiden in Betracht kommenden Enzymen kundgeben. In der Tabelle XVII sind die Eigenschaften der beiden Enzyme gegenübergestellt,* woraus wir sehen, dass, abgesehen von

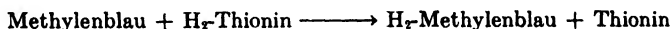
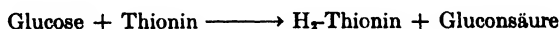
* Anmerkungen zur Tabelle XVII. Die mit * bezeichneten Angaben beruhen auf Befunden von Franke und Lorenz (25) sowie Franke und Deffner (24) und die übrigen sind nach Müller (57, 58, 59, 60, 63). Hinsichtlich der Verwendbarkeit der Akzeptorfarbstoffe findet man in den Angaben von Franke u.a. und von Ogura allerdings einige Unterschiede: so ist z.B. dem Toluylenblau von Franke u.a. eine positive, von Ogura dagegen eine negative Verwertbarkeit zugemessen. Beim Vergleich der einzelnen Akzeptorfarbstoffe kann man aber zwischen Verwertbarkeit und Unverwertbarkeit keine scharfe Grenze ziehen, denn wie von Ogura (noch nicht veröffentlichte Arbeit) neulich bewiesen, handelt es sich dabei nur um einen Unterschied der Reaktionsgeschwindigkeit, gar nicht aber um eine absolute thermodynamische Bedingtheit. Durch potentiometrische Untersuchungen hat Ogura nachgewiesen, dass die Reaktion $\text{Glucose} \rightarrow \text{Gluconsäure} + \text{H}_2$ einen irreversiblen Prozess darstellt, und ferner, dass dadurch auch diejenigen Redoxfarbstoffe wie Methylenblau, die mit Thunberg-Methode nicht mit merklicher Geschwindigkeit entfärbt, allmählich aber vollständig reduziert werden. Die thermodynamische Möglichkeit der Methylenblaureduktion lässt sich dabei durch folgende Experimente überzeugend beweisen: Setzt man dem Reaktionsgemisch: Methylenblau + Glucose + Glucosedehydrase eine kleine Menge von irgendeinem schnell reduzierbaren Farbstoff wie Thionin zu, so tritt innerhalb von etwa 50 Min. eine vollkommene Reduktion des Methylenblaus ein, was in dem Kontrollansatz ohne Thionin erst nach längerer Zeit stattfinden kann. Diese Erscheinung ist dadurch erklärbar, dass das Thionin, welches sowohl mit Enzym-Substrat-System wie auch mit Methylenblau

(1) dem Verhalten gegenüber Sauerstoff, (2) dem Dehydrierungsvermögen gegenüber Xylose und (3) der Reihenfolge der Dehydrierbarkeit der einzelnen Zuckerarten, eine schlagende Ähnlichkeit zwischen den beiden Enzymen besteht.*

Sehr ansprechend erscheint also die Mutmassung, dass die beiden Enzyme entweder denselben Proteinanteil und eine ähnliche aber doch etwas verschiedene prosthetische Gruppe, oder umgekehrt dieselbe Wirkgruppe aber etwas modifizierte Proteinanteile besitzen dürften, sodass dadurch auf jeden Fall ihr verschiedenes Verhalten gegenüber Sauerstoff zustande kommt. Verschiedenes Verhalten der beiden Enzyme gegenüber den Substraten scheint aber zunächst die Möglichkeit auszuschliessen, dass sie ein und denselben Proteinanteil besitzen. Neuerdings wurde von Müller (66) mitgeteilt, dass er im Präparat der "Glucoseoxydase" aus *Asp. niger* zwei verschiedene Enzyme mit ähnlicher Wirkungsweise nachgewiesen hat. Das eine soll die eigentliche, von ihm lange studierte "Glucoseoxydase," und das andere eine Glucosedehydrase sein, welche letztere, ähnlich wie das Enzym von Ogura, ganz anoxytrop ist, und in Gegenwart von 2,6-Dichlorphenolindophenol neben Glucose, Galactose und Mannose auch auf Xylose dehydrierend wirkt. Durch Erwärmung des Enzympräparates auf 60° soll diese anoxytrophe Glucosedehydrase, nicht aber die "Glucoseoxydase," zerstört werden. Auch solche Ergebnisse sprechen dafür, dass die Proteinanteile des Müller'schen und des

(Fortsetzung der Fussnote von S. 230)

schnell oxydoreduktiv reagieren kann, eine katalysierende Rolle bei der Wasserstoffübertragung spielt, nämlich:



Es wurde schon von mehreren Autoren festgestellt, dass die Reduktion der Redoxfarbstoffe durch ein Dehydrasen-System oder ein nichtenzymatisches Reduktionssystem oft um so schneller erfolgt, je höher die Normalpotentiale der betreffenden Farbstoffe sind, d. i. je steiler die Potentialgefälle zwischen oxydierenden und reduzierenden Systemen sind. Diese vom Standpunkt der kinetischen Theorie sehr interessante Erscheinung findet man am allerdeutlichsten in dem Fall von Glucosedehydrase (vergl. Barron und Hastings (2); Barron und Hoffman (3); Borsook (8)).

* Auf den Unterschied des pH-Optimums kann hier kein grosses Gewicht gelegt werden, weil, wie es wohl bekannt ist, die pH-Abhängigkeit des Enzyms je nach seiner Darstellungsmethode sowie den Versuchsbedingungen oft recht verschieden ausfallen kann. Bei verschiedenen Dehydrasen ist übrigens bekannt, dass beim Übergang von anaeroben zu aeroben Bedingungen das pH-Optimum oft nach der sauren Seite verschoben wird (vergl. z.B. Cook und Alcock (15)).

TABELLE XVII

EIGENSCHAFTEN DER MÜLLER'SCHEN UND DER OGURA'SCHEN GLUCOSEDEHYDRASE

		Glucosedehydrase von Müller	Glucosedehydrase von Ogura
Unterschiede	Herkunft	<i>Aspergillus niger</i> , <i>Pennicillium glaucum</i> u. <i>Citromyces</i> * (nicht vorhanden in <i>Asp. oryzae</i> u. <i>Asp. fumigatus</i> *)	<i>Aspergillus oryzae</i>
	Ausgangsmaterial bei der Darstellung	Alkohol-Äther-Fällung des Pilzpressaftes*	Extrakt des Pilzbreies mit alkalischem Phosphatpuffer
	Verwendbarkeit des Sauerstoffs als H-Akzeptor	+	—
	Dehydrierbarkeit des Zuckers	Glucose > Galactose > Mannose	Glucose > Xylose > Mannose > Galactose
	Dehydrierbarkeit von Xylose	—	+
	pH-Optimum	4.7–6.0; * 5.5–6.5	7.8
Gemeinsame Eigenschaften		Glucose wird zu Gluconsäure dehydriert.* Fructose und Arabinose werden nicht angegriffen. Coferment sowie Phosphat nicht notwendig. Verwertbare H-Akzeptoren:* Chinon, <i>o</i> -Chlorphenol-indophenol, Phenol-indo-2,6-dichlorphenol, <i>o</i> -Kresol-indophenol, <i>o</i> -Kresol-indo-2,6-dichlorphenol, Thionin. Nicht oder nur schlecht verwertbare H-Akzeptoren:* Cytochrom <i>c</i> , 1-Naphthol-2-Sulfonat-indo-2,6-dichlorphenol, Methylenblau, Indigotetrasulfonat, Nilblau. Einfluss des Giftes: Durch CN und Monojodacetat nicht gehemmt. Durch Urethan gehemmt.*	

* Aus den übrigen Zuckerarten sollten die entsprechenden Hexonsäuren bzw. Pentonsäure entstehen, obwohl deren direkter Nachweis bei enzymatischen Versuchen heute noch nicht erbracht worden ist. Knobloch und Meyer (35) haben neulich dargetan, dass *Aspergillus niger* aus Mannose Mannonsäure, aus Galactose Galactonsäure bildet, wofür offensichtlich die Wirkung von Glucosedehydrase verantwortlich zu machen ist.

Ogura'schen Enzyms nicht dieselben sein können. Franke und Déffner (24) haben neulich wahrscheinlich gemacht, dass die prosthetische Gruppe des

Müller'schen Enzyms wohl ein Flavin sein dürfte. Dass verschiedene Flavinenzyme mit Riboflavinphosphorsäure oder Riboflavin-Adenin-Dinucleotid je nach den Arten des Proteinanteils verschiedene Affinität gegenüber Sauerstoff aufweisen, ist heute eine allgemein bekannte Tatsache. Bei ihrem Versuch der Reindarstellung der Glucosedehydrase haben Franke und Deffner bemerkt, dass das u.a. durch Ammonsulfatfällung möglichst gereinigte Präparat bei weiterem Reinigungsversuch mit Aceton vielmehr an Wirksamkeit (gemessen durch O_2 -Aufnahme) einbüsst. Dabei nahmen die genannten Forscher an, dass durch die Acetonbehandlung eine teilweise Spaltung des Enzyms in Protein und prosthetische Gruppe herbeigeführt werde. Dies dürfte aber auch so zu deuten sein, dass dabei eine gewisse chemische Veränderung am Proteinanteil eingetreten sei, in dem Sinne, dass dadurch die oxytrophe Eigenschaft des Enzyms gestört wird. Leider ist von Franke und Deffner keine Angabe darüber gemacht worden, ob und wie das Verhalten des gereinigten Enzyms gegenüber den Akzeptorfarbstoffen durch Acetonbehandlung beeinflusst werde. Die nähere Analyse der stofflichen Natur der beiden, offenbar sehr nahestehenden Enzyme sowie die Aufklärung der Ursache der Verschiedenheit ihres Verhaltens gegenüber Sauerstoff ist mit grossem Interesse zu erwarten.

Wenn es anzunehmen ist, dass die anoxytrophe Eigenschaft der Ogura-schen Dehydrase nicht einen bei der Extraktionsprozedur entstandenen Artefakt, sondern eine wesentliche Eigenschaft des Enzyms darstelle, so muss gefolgert werden, dass bei ihrer Funktion in der Zelle ein gewisses Redoxsystem mit einem recht hohen Normalpotential als H-Akzeptor fungieren dürfte. Aus der noch nicht veröffentlichten Arbeit von Nagahisa und Ogura möchte ich hier vorausgreifend ein Ergebnis anführen, welches uns bezüglich der Frage der Wirkungsweise der Glucosedehydrase *in vivo* einige wichtige Aufschlüsse zu geben scheint. Aus den Zellen von *Aspergillus oryzae* konnten die genannten Forscher einen Redoxfarbstoff (oder ein Gemenge der Redoxfarbstoffe) extrahieren, der von dem Glucose-Glucosedehydrase-System glatt reduziert wird.* Während dieser Farbstoff an sich nur langsam autoxydierbar ist, wird er in Gegenwart von Hutipilzoxydase (Oxydase aus *Lactarius piperatus*) oder von Laccase aus Lackbaum schnell zu einem kirschrot gefärbten Farbstoff oxydiert. Mit solchen Oxydasen, dem betreffenden Farbstoff, Glucosedehydrase und Glucose kann man ein System konstruieren, in welchem die Glucose mit einer gros-

* Dieser Farbstoff wurde aus den Mycelien von *Aspergillus oryzae* durch Extraktion mit Aceton und Äther als eine ölige Substanz erhalten, die allerdings noch recht unrein ist. Die weiteren Reinigungsversuche dieses Farbstoffs sind jetzt im Gange.

sen Geschwindigkeit unter O_2 -Aufnahme oxydiert wird. In Figur 3 ist das Ergebnis eines solchen Versuches wiedergegeben. Natürlich darf man nicht voreilig darauf schliessen, dass dieser unbekannte Farbstoff, der höchstwahrscheinlich die Struktur von entweder Diphenol oder Phenylen-diamin oder aber Aminophenol besitzen dürfte, einen normalen Zwischenakzeptor bei der Tätigkeit der Glucosedehydrase *in vivo* darstelle, doch ist es immerhin sehr möglich, dass bei der Betätigung der Glucosedehydrase

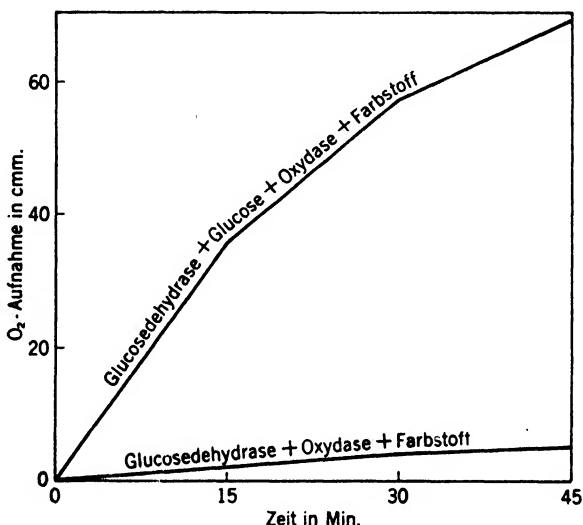


Fig. 3.—Konstruktion des oxytropen Systems aus der anoxytropen Glucosedehydrase (1.0 cc.), Glucose (M/4, 0.5 ccm.), Laccase (in Phosphatpuffer: M/2, pH 7.2; 0.2 ccm.) und Pilzfarbstoff (0.8 cc.). Bei folgenden Systemen wurde gar keine O_2 -Aufnahme beobachtet: Dehydrase + Glucose (mit oder ohne Oxydase), Dehydrase + Farbstoff (mit oder ohne Glucose). Nach Nagahisa und Ogura.

in der Zelle ein gewisses Redoxsystem mit einem recht hohen Normalpotential wohl unter Mitwirkung der darauf eingestellten Oxydase als Überträger beteiligt sei.

Es besteht kein Zweifel darüber, dass die Gluconsäurebildung, die von vielen *Aspergillus*-Arten bei Kultur auf Glucose bewirkt wird, entweder durch Glucosedehydrase nach Müller oder nach Ogura bewerkstelligt wird. Die in dieser Beziehung wiederholt diskutierte Frage, ob die Gluconsäure-

bildung eine normale Zwischenstufe beim respiratorischen Glucoseabbau darstellt oder nicht, ist aber ohne weiteres nicht leicht zu beantworten. Zur endgültigen Entscheidung dieser Frage müssen wir noch Vieles über die wechselseitigen Beziehungen zwischen Glucosedehydrase und anderweitigen oxydoreduktiven Enzymsystemen in der Zelle sowie über das weitere Schicksal der Gluconsäure wissen, welch letztere Substanz zwar im Stoffwechsel von *Aspergillus* gut verwertet wird, aber deren Umsatz bis jetzt noch nicht an Enzympräparaten nachweisbar gemacht worden ist.*

Was den Abbauprozess von anderweitigen C-Quellen anbelangt, die sich von der einfachsten Substanz wie Äthylalkohol bis zu äusserst komplizierten C-Verbindungen wie Eiweiss, Lipode u.a. erstrecken, so muss er—trotz der auffallenden Übereinstimmung, dass sie alle schliesslich zu Kohlensäure und Wasser oxydiert werden—ohne Zweifel, von Fall zu Fall, in äusserst mannigfaltiger Weise verschieden gestaltet sein. Nicht nur hinsichtlich der Atmungsvorgänge, sondern auch, ja in viel bedeutenderem Masse, bezüglich der synthetischen Vorgänge des Pilzes, an welchen augenscheinlich auch zahlreiche Redoxasen neben allen Arten von Hydrolasen beteiligt sein müssen, bleibt noch ein riesengrosses, beinahe ganz unbetretenes Forschungsgebiet, welches der enzymologisch-analytischen Untersuchung der Zukunft harrt. Die Probleme der synthetischen Leistungen der Organismen gehören überhaupt zu dem noch wenig beleuchteten Gebiete der Biochemie. Besonders in dieser Hinsicht stellt *Aspergillus* ein sehr geeignetes Forschungsobjekt dar, weil bei ihm der Aufbaustoffwechsel, wie schon gezeigt, immer so ausgiebig erfolgt, dass er oft ca. 80% des ganzen stofflichen Umsatzes ausmacht.

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* Die Dehydrase, die auf Gluconsäure dehydrierend wirkt, ist bis jetzt nur bei Hefen nachgewiesen worden (Müller (65)).

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CELLULOSE DECOMPOSITION BY MICROORGANISMS

By

A. G. NORMAN AND W. H. FULLER

Ames, Iowa

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I. Introduction

Cellulose is the major structural constituent of all plant materials, and indeed is probably the most abundant single organic compound known. Through the action of microorganisms it is decomposed in many natural processes under widely different conditions, in and on the soil, in lakes and rivers. It is digested by herbivorous animals and insects largely through the agency of microorganisms in the digestive tract. In its many industrial uses in the form of paper, fabrics, fibers and derivatives it is subject to attack by microorganisms. It is a potential source of useful products by controlled fermentation by microorganisms. In spite of these many circumstances under which cellulose may be attacked and decomposed microbially the list of authentic cellulose-decomposing organisms, although including representatives of various groups, is relatively short, and de-

tailed information as to the route and mechanism of decomposition and the enzyme systems involved, such as is possessed in the case of the dissimilation of monosaccharides, is very scanty. The conventional approach, which consists of pure cultural studies on a pure cellulose substrate, is apparently too narrow and too rigid, inasmuch as in most natural circumstances under which cellulose is attacked the active agent is a mixed population and the cellulose itself is in intimate association with other cell-wall constituents of different availabilities. Pure cultural-pure substrate studies are essential but can never be expected completely to illuminate the whole process of decomposition of cellulose as it occurs under natural conditions. It is the purpose of this review to examine the accumulated knowledge against the background of the broad problems to be solved.

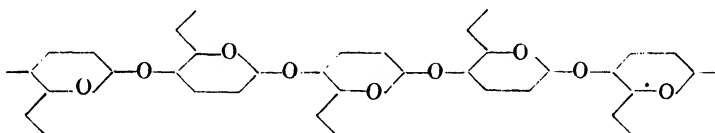
II. Chemistry of Cellulose*

1. *Pure Cellulose*

Cotton cellulose is ordinarily regarded as the typical cellulose and in fact, with mild purification to remove fatty material, closely approaches the simple glucose polysaccharide $(C_6H_{10}O_5)_n$. The general constitution of cellulose is fully established but there are important questions connected with its molecular architecture and form of aggregation as a macromolecule the answers to which have not yet been obtained. The building unit is glucose, which may be obtained hydrolytically from cellulose in almost quantitative yield. The kinetics of hydrolysis point to a β -glucosidic linkage uniformly throughout the cellulose molecule. Exhaustive methylation gives a derivative which on analysis is found to correspond closely to the trimethylated compound, and from which, on hydrolysis, 2,3,6-trimethylglucopyranose is obtained in almost quantitative yield. The presumption is, therefore, that the cellulose molecule consists of a long chain of β -glucose units uniformly linked together in the 1,4-position. It may be ascertained from steric models that glucose units linked in this manner give an almost straight chain if each alternate unit is turned through 180° , or, in other words, if the terminal carbinol group (C 6) lies alternately on one side of the chain and then the other. This gives the appearance of a repeating disaccharide unit, though there is no difference in the linkage between and within pairs. Cellulose is not in any sense to be considered as a polymer of the disaccharide, cellobiose, although cellobiose octacetate can be obtained from it by acetolysis in substantial but not the-

* A detailed bibliography of references supporting this section is not presented.

oretical yields. Preparations containing three, four and more glucose units have been obtained by controlled hydrolysis and again the uniformity of the linkage proved by identification of the methylated degradation products. Confirmation of the structure of the cellulose molecule was given by x-ray refraction data. Natural fibers are in fact thin bundles of molecular chains more or less uniformly oriented along the direction of the fiber. A periodicity or repeating unit of 10.25 Å is evident from photographs and this is known to be the length of two anhydro glucose units linked in the 1,4-position. The cellulose chain may therefore be represented diagrammatically as



Any simple chain must be terminated, and one end of such a poly-glucose chain would be expected to be reducing. Hydrolytic fragments of the cellulose molecule of variable length, known as cellulose dextrans, or sometimes cellodextrans, possess reducing properties, but in cellulose this property is ordinarily lacking or doubtfully detectable. This might be either because the chains are of enormous length or because some modification of the end-group, such as oxidation to carboxyl, takes place. The length of the cellulose chains and therefore the molecular weight of cellulose are highly controversial. Physical methods based on the viscosity of solutions of cellulose derivatives in organic solvents, and end-group assay methods based on the quantitative separation of 2,3,4,6-tetramethylglucose from the 2,3,6-trimethylglucose obtained on hydrolysis of fully methylated cellulose, have given variable results. It is, however, quite apparent that the chain length of cellulose may be substantially changed by comparatively mild treatment, and in any case there is no reason why cellulose from different sources should necessarily have the same chain length. The assumption has been made in interpreting the results of physical measurements that simple chain molecules are involved even though the chains are composed of six-membered rings and are not simple as in a hydrocarbon. It now appears probable that a certain amount of looping or cross-linkage between chains occurs, and this, if true, will render questionable and perhaps invalidate some previous calculations. By the end-group assay method the average lower limits of size of the cellulose molecule have been indicated to lie between 100 and 200 glucose units, though by some form of aggregation the macro-molecule may be much larger in

size. However, methylations in air and in an inert gas have given entirely different results, and products containing varying numbers of glucose units, as deduced from osmotic pressure and viscosity measurements, have been found, in the absence of air, to have no end-group capable of methylation. The suggestion has been made, therefore, that although the cellulose chain is broken with consequent reduction in molecular size, the exposed end-groups are involved in some sort of recombination. Haworth rejects the possibility of simple head to tail linkage with an adjacent cellulose chain, and believes that chains are normally cross-linked laterally at various points along their length, the cross-linkage perhaps occurring as frequently as every 25-30 glucose units. When breaks in the chain occur they would take place at these points. No satisfactory information as to the nature of those cross-linkages has been obtained. It is unlikely that simple primary valency bonds are involved because in that event significant amounts of dimethylglucose should be found among the hydrolysis products of methylated celluloses, and this is rarely the case if the original methylation is complete. Complete methylation, however, is more difficult than might be expected, and it is possible that some of the hydroxyl groups on contiguous chains are abnormal, perhaps as a result of drying. Whatever may be the ultimate explanation, it seems probable that the structure of cellulose is incompletely represented by single terminated chain molecules of great length, and that the macro-molecule is an aggregate of such thread-like chain molecules, the whole complex together giving a structure of great stability and longitudinal strength. There may, in fact, be no well-defined cellulose macro-molecule, but instead an interlocked system of cellulose chains.

In cellulose fibers and the cell wall of plant tissues micellae composed of bundles of cellulose chains are recognized, and these, and not individual cellulose chains, are in fact the structural units. The micellae are not necessarily well-defined discrete units. In fibers the micellae are usually arranged in a spiral at a slight angle to the direction of the fiber. Cell walls, on the other hand, may be composed of laminae, the micellae of which may be set in different directions in each sheet, and the orientation of which is far from perfect.

2. Cellulose as a Cell-Wall Constituent

Cellulose is the structural component of plant materials, and, except in the cotton fiber, is always found in the most intimate association with other cell-wall constituents from which separation without some change is

probably impossible. While it is unlikely that cellulose is chemically linked to its associates, primarily the polyuronide hemicelluloses and lignin, or that "compound" celluloses occur, nevertheless the normal structure is such that interpenetrating systems are found. The cellulosic fabric composed of oriented micellae is, as it were, interwoven with amorphous systems composed of lignin or polyuronide hemicelluloses, each of which may be removed by appropriate treatments leaving the others intact and still exhibiting the form of the tissue.

It is particularly important to notice that the structural cellulosic fabric of most plants is not composed exclusively of the glucose-polysaccharide typified by the cellulose of the cotton fiber. The cellulose building mechanism of the ordinary plant apparently produces a product far from uniform although with the same general steric and chemical pattern as cotton cellulose. In cotton the individual molecular chains are of much the same length and the aggregates form a relatively homogeneous system. In most plant celluloses there may be a fraction more or less corresponding to cotton cellulose in molecular dimensions but this is associated sterically with much shorter chain material, not necessarily composed only of glucose units. Almost all plant celluloses contain xylan, in amounts sometimes as much as 25 per cent. The gymnosperms in addition may contain mannan, though in such cases the xylan content is usually near 5 per cent. These associated polysaccharides, now known as cellulosans, are not impurities and have no independent existence. Their molecules are oriented in the same direction as the cellulose molecules, participate in the micelles and presumably the same forces are operative in their retention. The individual units of a xylan or mannan chain occupy the same space longitudinally as the glucose units in the cellulose chain, and xylan indeed differs from the latter only in lacking the projecting carbinol units. The chain length of the cellulosans is, however, much less than that of the true cellulose chains and as a result they may be extracted by alkali more or less completely, depending on the concentration employed, or hydrolyzed by hot dilute acids, which fail appreciably to attack the long cellulose chains. The residue after these treatments cannot be said to be a typical plant cellulose. When wet, it has a structure honeycombed with cavities and faults. On drying, adjacent chains may draw together to some extent, as indicated by the fact that the crystallographic picture of certain fibers from which the cellulosans have been removed hydrolytically has been found to be improved. Filter paper, so often regarded as a typical cellulose, in fact consists of wood cellulose that has been treated in such a manner.

III. Biochemistry of Cellulose Decomposition

Cellulose is completely insoluble in water and obviously therefore to accomplish cellulose decomposition any organism must possess an extra-cellular enzyme system capable of producing some soluble product that can pass into the cell. The insolubility of cellulose alone is sufficient reason why in pure culture-pure substrate studies decomposition is often slow in getting under way unless a heavy inoculum, undesirable in quantitative studies, is made. Knowledge of the enzyme system effecting the primary attack on cellulose is extraordinarily scanty in view of the widespread occurrence of cellulose decomposition in natural processes. The conventional view is that an exo-enzyme brings about hydrolysis of the cellulose molecule with consequent liberation of glucose, which is subsequently dissimilated intercellularly, the products formed therefrom varying with the nature of the organism. If a simple chain molecule represents the structure of cellulose, this enzyme should be a simple hydrolytic system capable of splitting off glucose units from the long cellulose chains. This might be accomplished progressively by removal of the terminal unit and gradual chain shortening, a process which would not necessarily be marked by the production of cellulose dextrans in any appreciable amounts. The strong probability that some type of cross-linkage between chains exists, together with the knowledge that comparatively mild chemical treatments bring about breakage of the chain into fragments that are still comparatively long make it unlikely that the hydrolytic system is as simple as pictured above. It is not known whether cellulose dextrans are produced in the enzymic attack on cellulose but there seems to be some indication that two types of hydrolytic systems may be concerned, one accomplishing the primary attack, perhaps breaking cross-linkages or accomplishing substantial chain-shortening, and the other bringing about the subsequent hydrolysis of these fragments. The evidence for this is the observation that insoluble dextrans prepared by controlled cold acid hydrolysis of cellulose are apparently utilized more readily, and by many more organisms than will develop on untreated cellulose.

Probably arguing by analogy with starch, from which the amylase system splits off the disaccharide maltose, it has been suggested that in the hydrolysis of cellulose cellobiose may be produced, and indeed Pringsheim (1) claimed to have demonstrated its production. There is no obvious structural reason why alternate glycosidic linkages should be preferentially hydrolyzed and cellobiose formed, but it must be admitted that from chemical considerations alone the same is equally true of starch, the linkages of

which are uniformly of the 1,4 α -glycoside type. Sterically, however, the situation is entirely different because in the branched starch molecule the relatively short chains are coiled, which may result in some emphasis of the disaccharide grouping, whereas in cellulose the chain is straight. The production of cellobiose from cellulose cannot be entirely ruled out as a possibility, however, because the disaccharide grouping might perhaps be considered to be emphasized in cellulose by the fact that each glucose ring is turned through 180° with respect to its neighbor.

Pringsheim (1) postulated the existence of two hydrolytic enzyme systems, cellulase and cellobiase, the former producing cellobiose from cellulose and the latter hydrolyzing the disaccharide to glucose. By the addition of antiseptics the growth of the organisms in a vigorous thermophilic fermentation was inhibited and reducing substances accumulated. These were identified as cellobiose and glucose, though the identification of the former was not beyond question. Similar results were obtained by raising the temperature of a thermophilic culture to such a point that growth was checked, but not so high that these enzymes were inactivated. The inactivation temperature of the cellobiase system was said to be lower than that of cellulase, so that by adjusting the temperature of the particular culture between 67° and 70°, cellobiose accumulation occurred without hydrolysis to glucose. It is somewhat surprising that the organisms should have separate extracellular enzyme systems of this nature. Cellobiose is easily soluble in water, and might be expected to enter the cell without previous hydrolysis to glucose. These observations remained long unconfirmed though often quoted. Glucose, identified by its reducing properties and characteristic osazone, was found in a thermophilic cellulose fermentation that had been checked by the addition of toluene and incubation at 37° instead of 65°, but no cellobiose could be detected (2). In extensive experiments with a number of mesophilic cellulose organisms similar results were obtained by Kalninš (3). Two species, *Bacterium protozoides* and *B. bosporum* produced soluble reducing substances from cellulose in small amounts under ordinary conditions. In the case of a number of species of the genus *Vibrio* accumulation occurred either when vigorous aerobic cultures were sealed to limit the oxygen supply, or when the incubation temperature was raised above the normal growth range. The maximum concentration of reducing substances found was 0.5 per cent calculated as glucose, and glucose was in fact the only substance to be identified. Dissolution of cellulose continued slowly in the sealed cultures.

The results obtained by Simola (4) in a detailed study of two aerobic spore-forming cellulose organisms named by him *Cellulobacillus myxogenes*

and *Cellulobacillus mucosus* support Pringsheim's contention of the existence of two distinct steps: cellulose \rightarrow cellobiose, and cellobiose \rightarrow glucose. Both glucose and cellobiose were identified by reducing values, specific rotations and m. p. of the osazones. Further, the disaccharide was shown to be hydrolyzed to glucose by the addition of emulsin. The activity of these enzymes in phosphate buffers was shown to be greatest between pH 5.0 and 6.0. Complete inhibition took place at pH 8.5. The optimal temperature was in the neighborhood of 37°. Cell-free solutions, obtained from toluene-treated fermentations after filtration through a Berkefeld filter, were shown to exhibit hydrolytic activity. Such preparations exhibited maximum activity in the hydrolysis of cellobiose to glucose between pH 4.5 and 5.5 and at 37°. The optimal pH range and temperature for these enzymes are not dissimilar to those given for cellulose-dissolving preparations from the gut of the snail (6).

Pochon (5) similarly obtained evidence of the accumulation of reducing bodies on the addition of toluene to a vigorous culture of *Plectridium cellulolyticum*. Glucose was partially identified and presumptive evidence of the presence of cellobiose given.

By preventing access of oxygen to well-established cultures of *Sporotrichum carnis* on cellulose or by the addition of toluene or iodoform Vartiovaara (7) demonstrated the accumulation of a reducing substance. The major part was considered to be glucose since only small increases in reducing power resulted from dilute acid hydrolysis. The presence of cellobiose could not be ascertained, nor was the characterization of glucose direct. Reducing substances were not found in normal aerated cultures, though water-soluble substances from which reducing sugars could be obtained on dilute acid hydrolysis were detected. This interesting observation was not carried further. It is hardly likely that the water-soluble substance was of the nature of a cellulose dextrin because of the lack of reducing power, and more probably it was of a synthetic nature akin to the polysaccharides produced by many fungi from sugars.

The existence of an extracellular hydrolytic enzyme system capable of producing either glucose or cellobiose from cellulose may be taken as proved, but what is lacking is any information as to the intermediate steps. Is there ordinarily production of cellulose dextrans, or are the soluble di- or monosaccharides the first recognizable products? Although it is not known how many glucose units may be linked together and still have the property of ready solubility in water, the number is certainly more than two and may be as great as 10-15. If the hydrolysis is such that short chain dextrans are formed, there is the possibility that these might be able

to pass into the cell, and that subsequent breakdown might then be intracellular. Complete extracellular hydrolysis is not essential. The observation that certain of the more specialized cellulose organisms are inhibited by relatively low concentrations of glucose might suggest that the dissimilation does not normally proceed through this sugar. The organism now termed *Cytophaga hutchinsoni* was reported to be completely inhibited by glucose concentrations as low as 0.1 per cent and by cellobiose at 1.0 per cent (8). The action on cellulose of other more versatile organisms was inhibited by glucose concentrations of 1 per cent and reduced at 0.5 per cent (3). The latter, however, might easily be accounted for in that the mass action of the accumulated soluble product, glucose, might prevent its further production from the insoluble material, cellulose.

A simple hydrolytic route of cellulose decomposition is not the only possibility, and the oxidative theory put forward by Winogradsky (9) must be examined. He noted certain similarities between the properties of filter paper attacked by cellulose organisms and chemically produced oxycelluloses. He suggested that terminal carbinol groups might be oxidized to aldehydic groups and that these and even other alcohol groups might further be oxidized to carboxyl groups. Cellulose attacked by bacteria was observed to have acidic properties, to give a yellow color with alkali and to be partially soluble in alkaline solutions, to be more active in the retention of dyes such as methylene blue, but not to exhibit reducing power with Fehling's solution. Only in the last characteristic was there an apparent divergence from the properties of the chemical oxycelluloses, but in no sense was this general similarity direct proof that oxycellulose is a primary product of bacterial attack on cellulose. The oxidative theory was apparently accepted by Kalniņš (3) and by Loicjanskaja (10) who attempted to support it by chemical evidence. The latter allowed small squares of filter paper to be partially attacked by *Cytophaga*, analyzed the residues for uronic content and furfuraldehyde yield and determined before and after hydrolysis the reducing value of the alkali-soluble fraction. Both the uronic content and furfuraldehyde yield increased with extent of decomposition, and these increases were accepted as evidence of an oxidative attack. Cellulose attacked by *Cytophaga* becomes mucilaginous, and indeed in the original description of this organism this material was described as of a pectic nature. From cellulose suspensions extensively decomposed by *Cytophaga*, Walker and Warren (8) isolated a gum or mucilage by alcohol precipitation after removal of the residual fibers. This they showed to be of an acidic nature, and to yield furfuraldehyde and carbon dioxide when treated with 12 per cent hydrochloric acid as do all poly-

uronides. Because of its low reducing power, they considered this gum to be an acidic type of oxycellulose, and maintained that it represents an intermediate stage in cellulose breakdown. They state, "It would seem that the primary alcoholic groups of the cellulose molecule are oxidized to carboxyl throughout the length of the chain; that the consequent instability causes (or renders easier) fragmentation of the large molecule, and that the partially oxidized fragments are further attacked by the organism, but that the attack is not carried to completion. To account in this manner for the occurrence of the mucilage implies that any given molecule of cellulose is only partially consumed which we find easier to believe than that a certain proportion of the oxidized cellulose is completely consumed and the remainder not further attacked."

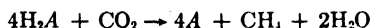
Although the observations of Loicjanskaja (10) and Walker and Warren (8) have been confirmed (11), their interpretations of the data as supporting the oxycellulose theory of attack are not justified. The postulated oxidative process might oxidize the terminal carbinol groups at random along the chain or more improbably might wholly transform a cellulose molecule into a polyglucuronide. In neither case is there any reason to believe that the oxidation would of itself cause immediate chain-splitting to such an extent that all the oxidized fragments would be water-soluble. Part of the uronic groups should therefore be found in the residual fibers upon separation from the gum or mucilage. Moreover, an oxidative attack presupposes the existence of an oxidative exo-enzyme system, which is without precedent. The postulated product, oxycellulose, is water-insoluble, and would still be unavailable to the organism. No energy could be derived by the cell until sufficient chain-splitting had occurred to render the fragments water-soluble, and if this could take place there would seem to be no obvious reason why an insoluble oxycellulose should accumulate. It was found that the residual cellulose was not more oxidized than initially and that in fact uronic groupings introduced by chemical means were actually preferentially removed (11). The characterization of oxycellulose was shown to be incomplete, and the products so described to be microbial gums of a polyuronide nature containing both uronic and pentose groupings.

The conclusion has to be reached that there is little authentic information as to the primary attack on cellulose, but that it is most probably of a hydrolytic nature. Colonies of cellulose bacteria on agar plates containing regenerated cellulose or dextrans are often surrounded by a small cleared zone, but the area so cleared is usually very restricted by comparison, for example, with that produced on starch agar plates by most organisms ca-

pable of developing on starch. It seems likely that exo-enzyme production is only stimulated by direct contact with the fiber, as might perhaps be expected in the case of a wholly insoluble substrate. Fibers undergoing decomposition are, when examined, seen to be almost invariably packed with microbial cells, or, in the words of Winogradsky, "littéralement tapis-sées" (9).

The later stages of cellulose decomposition are not much more satisfactorily understood than the nature of the primary attack. Various products have of course been described but accurate balance sheets are few, and indeed the precise products given by some of the most commonly recognized cellulose bacteria cannot be found in the literature. In general it seems that the aerobic mesophilic bacteria effect extensive oxidation, the major product being carbon dioxide. For example, two-thirds of the carbon removed from cellulose decomposed by *Cytophaga* was recovered as carbon dioxide, and almost all of the remainder could be accounted for in the gum synthesized by this organism (8). No more than a trace of acid, either volatile or non-volatile, could be detected. None of the many species of *Vibrio* studied by Kalniņš (3) produced acid from cellulose. The two aerobic spore-formers described by Simola (4) yielded about 10 per cent volatile acid, consisting of acetic and formic, the former always being considerably in excess of the latter. In addition a small amount of an unidentified non-volatile acid and less than 1 per cent alcohol were obtained. No acid was produced by any of the cultures studied by Norman and Bartholomew (11). On the other hand, anaerobic organisms, as might be expected, give substantial amounts of acidic products and some alcohol from cellulose, and in fact numerous attempts have been made to develop large-scale fermentation processes in which the yields would be high enough to make commercial development possible. So far this has not been accomplished. Long ago Omelianski (12) demonstrated the production of high yields of fatty acids, primarily acetic and butyric, together with carbon dioxide, and either hydrogen or methane, by the cultures described as *Bacillus methanigenes* and *Bacillus fossilicularum*, the absolute purity of which, like that of so many of the cellulose-decomposing anaerobes, is a matter of some doubt. Indeed it is now considered unlikely that methane is a primary product of cellulose decomposition. In all probability methane is derived from the ultimate dissimilation products of cellulose in a secondary fermentation by methane organisms, and its appearance may be taken as an indication that the cellulose culture is impure. Barker (13) has given proof that the origin of the methane lies in a transfer of hydrogen from one of the primary fermentation products to carbon dioxide,

in this confirming the van Niel hypothesis that the following equation is applicable:



The products usually given from cellulose by anaerobic organisms are acetic, butyric and lactic acids, ethyl alcohol, carbon dioxide and hydrogen, though traces of other alcohols and acids may also be found. It is a characteristic of many of these organisms that the yields of any particular constituent are not very stable. Since some of the products are acids, and acidity limits growth, some means of controlling progressively the pH of the fermentation has to be adopted. Frequently this is done by addition of calcium carbonate, which cannot be well distributed throughout a cellulose suspension, and which is rarely wholly satisfactory. Variations in yields are therefore frequently due to incomplete control of the reaction of the fermentation or to culture impurity. With the same inoculum the nature of the cellulose has been shown to affect appreciably not only the extent of fermentation but also the ratio between products (14, 15, 16). The yield of volatile acid, however, is usually in the neighborhood of 50 per cent of the cellulose fermented, with acetic acid predominating.

The most vigorous thermophilic cultures are usually composite, and separation and the establishment of culture purity are a matter of considerable difficulty. Perhaps the most comprehensive study of such a composite culture was made by Snieszko, who by an ingenious process of recurrent pasteurization effected the separation of a vigorous thermophilic culture into three organisms, two of which, although incapable of attacking cellulose, speeded up the rate of fermentation accomplished by the third, which by itself was much slower and less effective (17, 18, 16). The pure culture yielded from cellulose about 55 per cent acetic acid and 11–15 per cent ethyl alcohol. When, however, the two associated facultative aerobes were present, the yield of ethyl alcohol was low, and, moreover, a high percentage fermentation of cellulose was usually correlated with a low yield of acidic products. On glucose or starch the composite culture gave rise to considerably more non-volatile acid and alcohol. Similar products were given by the Khouvine organism *Bacillus cellulosae dissolvens*, a strict anaerobe with optimum temperature between 38° and 50°. Impure cultures were reported to be far more vigorous than the pure culture, which was incapable of utilizing other carbohydrates. Woodman and Evans (19) compared crude cultures of cellulose organisms from sheep rumen incubated at 65° and 37°. The nature and proportions of

the acids obtained varied from culture to culture as might be expected. Much greater amounts of lactic acid were detected at 65° than at 37°, but the reverse was true with respect to pyruvic acid which appeared briefly at an early stage. There was of course no assurance that precisely the same organisms were active at both temperatures.

The balance of acids given by the thermophilic anaerobe *Plectridium cellulolyticum* is unusual in that formic was found to predominate. Of the carbon lost 64 per cent was recovered as volatile acid, 80 per cent of which was formic, 16.6 per cent acetic and the remainder propionic (5).

Little is known of the products of decomposition of cellulose by fungi or actinomycetes and in fact pure cultural studies have been extremely few. From sugars many fungi produce appreciable quantities of non-volatile acids, particularly oxalic, citric and gluconic acids, and rarely more than traces of volatile acids. However, none of these have been reported as products from cellulose, despite the probability of their formation. In detailed studies of the metabolism of selected soil fungi, Vartiavaara (7) found no significant change in the pH of cultures which accomplished extensive decomposition of pure cellulose. By far the most obvious, and from the agronomic point of view, the most important, product is synthesized microbial substance. It has already been mentioned that some of the aerobic mesophilic bacteria produce appreciable amounts of gum, but the product in the case of fungi is fungal cell-substance. As much as 50 per cent of the carbon of the cellulose utilized may be used structurally in the mycelium synthesized. This implies a substantial demand for nitrogen, which if supplied in the inorganic form will be temporarily immobilized in the fungal tissues in the form of nucleoproteins and other organic nitrogenous complexes. The ratio between cellulose decomposed and nitrogen immobilized in this way has been found to be 30-35 to 1 (20). That is to say, in the decomposition of 100 units of cellulose by fungi about three units of nitrogen will be required. The requirements of mixed aerobic cultures including both bacteria and fungi are, however, lower, partly because the synthetic activities of bacteria are at a lower level and partly because synthesized fungal tissue does not accumulate to the same extent but is itself used as a carbon source by bacteria.

IV. Cellulose-Decomposing Organisms

Under this heading will be discussed the characteristics of some of the groups of cellulose-decomposing organisms, as revealed by studies with

isolated cellulose as the substrate. Many different types of organisms have been shown to possess the ability of developing on cellulose, but in some way the impression has prevailed that, in general, cellulose-decomposing ability is a property of highly specialized organisms. While it is true that certain of these exist and are particularly interesting, it is improbable that they are dominant in natural processes involving the decomposition of cellulosic material. Many of the cellulose organisms are morphologically variable, and this fact, together with the inapplicability of the ordinary plating techniques to an insoluble substrate such as cellulose, make the establishment of absolute culture purity a matter of considerable difficulty and anxiety.

1. *Cultural Methods*

The numerous methods proposed for the isolation and purification of cellulose-decomposing bacteria fall into two major categories, namely: methods using some form of purified natural cellulose, and methods using regenerated cellulose, that is, cellulose that has been dissolved and reprecipitated, thereby losing in great measure the orientation of the micelles.

Most of the early workers obtained crude cultures of cellulose organisms directly from decaying plant tissue, from filter paper or linen cloth inserted into the soil (21) or by placing small particles of soil in nutrient solutions to which cellulose had been added, usually in the form of filter paper. The last method is essentially that used by Omelianski (22) in his pioneer work in this field. Relatively pure cultures were obtained by repeated transfers to fresh sterilized medium. A modification of Omelianski's method using filter paper strips was proposed by Dubos (23), for the isolation and enumeration of cellulose organisms, particularly those that are strictly aerobic. He claimed that the nutrient solution employed inhibited the growth of fungi and that pure cultures can frequently be obtained by repeated dilution and transfer as soon as growth is evident.

In the isolation of a thermophilic culture, Tetrault (24) made use of a solid medium, consisting of 0.8 per cent agar, to which nutrients and finely ground filter paper were added. He also employed rounds of filter paper imbedded in solid nutrient agar. Solid and semi-solid nutrient agar in various combinations has been used by others, (18, 16, 25) to some extent in the isolation of thermophilic bacteria, and to a greater degree in the isolation of aerobic mesophilic organisms (26, 27, 28).

The use of silica-gel plates upon which filter paper is laid or a cellulose suspension is spread, has been advocated (29, 30, 31). Although this method has been suggested (31) for obtaining pure cultures, it is more satisfactory for demonstrating the presence of cellulose-decomposing organisms than for the isolation of pure cultures.

The first to use regenerated cellulose for the isolation and purification of cellulose-decomposing organisms were Kellerman and McBeth (32). They used a cellulose agar medium adapted to the conventional dilution plating technique. The cellulose consisted of filter paper dissolved in cuprammonium solution, re-precipitated by acid and carefully washed. Such re-precipitated cellulose is considerably changed physically and

structurally, though there is probably no extensive reduction in molecular size. Other workers employed cellulose re-precipitated after solution in zinc chloride (33) or ferric chloride (34). This, however, is more strongly hydrolytic, particularly if the temperature is not carefully controlled. Scales (35) suggested a sulfuric acid method that is simpler than those just mentioned, but the effects of which are less well understood. Filter paper is dissolved in about 60 per cent sulfuric acid solution at a temperature of 60–65° and immediately precipitated by pouring into cold water. The precipitated cellulose, which appears as a structureless gel, is quite satisfactorily incorporated in an agar medium or in silica gel (3). Chemically it might more properly be described as hydrocellulose, or cellulose hydrate, the crystallographic picture and water relationships of which are entirely different from those of normal cellulose.

Recently cellulose dextrans have been used in the isolation and purification of some aerobic mesophilic bacteria. The method of preparation consists of hydrolyzing cellulose in cold 72 per cent sulfuric acid, precipitating water-insoluble fractions by dilution with cold water at various time intervals and an alcohol-insoluble fraction by addition of excess ethanol to the combined filtrates after neutralization and concentration. The latter fraction dissolves in water and gives a clear medium with agar. The water-insoluble dextrans are washed free from acid, concentrated down and the suspensions made up into cellulose agar media. These media are well adapted to the dilution plating technique and have the added advantage of being opalescent so that active cellulose decomposers developing on them can be easily identified by the appearance of a clear halo around the rather restricted colonies.

Cellulose derivatives such as the esters or ethers have been little used, largely because, in general, they are not readily attacked by microorganisms. However, Krzemieniowska (36) discovered that *Cytophaga* could be grown on cellophane, but she was unable to obtain pure cultures using cellophane as a substrate. Notwithstanding this disadvantage the use of cellophane furthered morphological studies of this group.

2. *Aerobic Bacteria*

Prior to the work of Van Iterson, Jr. (37), it was not certain that cellulose utilization was a property of any aerobic bacteria. He obtained an aerobic culture which he termed *Bacterium ferrugineum* the purity of which seems to have been doubtful. It was not until Kellerman and his associates (32, 38) developed the regenerated cellulose agar medium that pure cultures were certainly obtained. These organisms were first described under the genus *Bacillus*, but later nearly all were transferred by Bergey to the new genus *Cellulomonas* (39). Most of them are small rods with rounded ends, either non-motile or motile with peritrichous flagella. Growth on ordinary culture media is often not vigorous, yet a wide range of carbon and nitrogen sources may be utilized. Some of the forms are chromogenic. Over twenty species of this genus are listed in Bergey's Manual (40). Additional studies of some of those organisms were made by Bradley and Rettger (41), who stress the lack of specificity of the cul-

tures and the relatively limited extent of cellulose decomposition accomplished by them under laboratory conditions.

Of the mesophilic aerobes most attention has been centered upon the cytophagas. This is due partly to their strict adaptation to cellulose and partly to their remarkable morphology. Van Iterson (37) appeared to have worked with organisms belonging to this group. The type species was isolated by Hutchinson and Clayton (42) and named *Spirochaeta cytophaga*. Young cultures on filter paper consisted of long, thin, flexuous rods, tapering or pointed at the ends, but in older cultures a large coccus or sporoid was invariably present. Separation was not accomplished by dilution procedures, and it was considered that these two forms represented different periods in the life cycle of the organism. The cells were motile though not flagellated. It was soon recognized that this organism was improperly described as a spirochete. Winogradsky (9) in an extensive study of aerobic cellulose-organisms obtained a number of cultures similar to the above but lacking the sporoid or coccus. He contended, therefore, that the coccus was a contaminant and continued study only on those cultures from which they were absent. On the basis of pigmentation, and to a lesser extent size, five species were described and a new genus, *Cytophaga*, proposed, of which the type species, *Cytophaga hutchinsoni*, was considered to be identical with the Hutchinson and Clayton culture without the coccus. Krzemieniewska (36) designated the cocci as microcysts and maintained that species exist with and without microcysts. The cultures of Hutchinson and Clayton, and Winogradsky were only capable of utilizing cellulose but species have been described that are far less specific (43).

Another interesting aerobic organism was described under the name of *Microspira agarliquefaciens* (44). Agar was liquefied and decomposed, as well as cellulose attacked, and other simpler carbon sources were utilized. This organism probably should be included under the genus *Vibrio*. A somewhat similar organism, *Vibrio amylocella* (45), possesses the property of decomposing cellulose, and also of liberating glucose from starch or starch-dextrin if the source of nitrogen supplied is ammonium chloride. The accumulation of glucose is related to the development of acidity which checks the growth of the organism. It does not grow on media containing beef extract, but will slowly utilize various sugars in low concentrations if supplied with nitrate. Winogradsky (9) set up a new genus, *Cellvibrio*, and described two species, one, *C. ochraceus*, producing a bright ochre-yellow pigment on filter paper, apparently being limited in its activities to cellulose, and another, *C. flavescens*, less restricted in its utiliza-

tion of carbon compounds but still only growing feebly on starch and glucose. Later two other species were added by Stapp and Bortels (46), both growing well on starch and differing mainly in pigment formation and vigor of growth on glucose and sucrose. Another new genus, *Cellfalcicula*, was proposed to include short pointed rods or spindles not over 2 μ in length (9). The three species described were said to make either feeble growth or no growth on peptone, glucose or starch.

An extensive investigation by Kalninš (3) resulted in the description of 48 strains of aerobic bacteria, classified into three genera and 17 species. Twelve of the species were curved rods belonging to the genus *Vibrio*, four belonged to the genus *Bacterium* and one to the genus *Bacillus*. Filter paper strips in nutrient solution were disintegrated at the surface of the liquid in two to five days. Many sugars were utilized by these organisms, which are not in any sense specialized in their activity on cellulose. Two aerobic spore-forming bacteria, similarly not specific in the utilization of cellulose, were studied exhaustively by Simola (47) and described as *Cellulobacillus myxogenes* and *Cellulobacillus mucosus*. Another spore-former, somewhat similar in properties but active in the lower thermophilic range, was described by Coolhaas (25) as *Bacillus thermocellulolyticus*. Growth on sucrose agar was less vigorous than on starch or cellulose agar. Other thermophilic organisms active under aerobic or anaerobic conditions alike were found, in fact, to be strict anaerobes with associated facultative forms. One such culture, believed at the time to be pure, was described by Viljoen, Fred and Peterson (28) and given the name *Clostridium thermocellum*.

3. Anaerobic Bacteria

Some of the mesophilic aerobic cellulose bacteria are capable of carrying on their activities also under anaerobic conditions. Many of the organisms isolated by Kellerman *et al.* (32, 38), are facultative, though not vigorously so. The same is true of the two species of *Cellvibrio* described by Winogradsky (9). Most of the true anaerobes studied fall into the thermophilic group. *Clostridium cellulosolvens*, isolated from horse dung by Cowles and Rettger (48), grows well at 37°. The rods are slightly curved with terminal spores. Dextrin, starch and some pentose sugars were utilized, as well as cellulose, but glucose was not attacked, nor did the addition of glucose to a cellulose fermentation inhibit or reduce the decomposition of the cellulose as is so often the case. The Khouvine organ-

ism, *Bacillus cellulosaе dissolvens* (49), isolated from the digestive tract of man is a strict anaerobe capable of utilizing cellulose as the only carbon source, and growing in the temperature range of 35–57°. Another organism active within this general range but less specialized in character has been isolated by Pochon (5) from the paunch of ruminants, and described as *Plectridium cellulolyticum*.

The true thermophilic anaerobes have been studied mostly with a view to the development of an industrial fermentation process to obtain acids or alcohol from cellulose. Crude cultures accomplishing very vigorous and rapid decomposition of cellulose at 65° can be obtained readily from dung after a few transfers. To obtain bacteriologically pure cultures, or indeed to establish unequivocally the purity of any culture, has proved to be a matter of great difficulty as shown by the series of investigations at Madison, Wis. (28, 24, 27). Snieszko (17) showed that carefully "purified" thermophilic cultures may still be composite, and effected the separation of such a culture by repeated pasteurization in broth permitting the germination of the spores of the associated forms, but in which an obligate cellulose fermenter would not develop. The true cellulose organism proved to be morphologically variable, with pronounced terminal spore formation, and to be an obligate anaerobe resembling in most respects the Khouvine organism and the obligate thermophilic bacteria described by Paine (50). The associated forms were facultative anaerobes, markedly pleomorphic and quite distinct.

4. *Classification and Systematic Position of Cellulose Bacteria*

An adequate system of classification and nomenclature for the cellulose bacteria is urgently needed. At present the situation is chaotic and is becoming worse as additional organisms are isolated and studied. It will be apparent from the survey above that two general classes of cellulose bacteria exist, obligate cellulose decomposers, and more versatile organisms capable of utilizing other carbon sources. All degrees of versatility may exist. Non-cellulosic substrates may be used rather feebly, so that cellulose appears to be the preferred energy source, or vigorous growth may occur on non-cellulosic sources, and cellulose be only slowly attacked. The crux of the matter, then, is whether the property of decomposing cellulose shall be regarded as so outstanding and distinctive that organisms having this ability shall be set off from other like organisms, similar in morphology or general fermentative characteristics, or both. Such a

separation, if generally followed in systems of classification in bacteriology, might lead to great confusion in certain groups. As far as the cellulose organisms are concerned, no consistent policy has been followed. Moreover, a secondary point that arises is how ability to utilize cellulose shall be recognized. It is highly probable, if not certain, that organisms exist which are capable of utilizing the cellulose of plant materials where it is associated with other more available constituents, and yet which are incapable of attacking filter paper strips or suspensions of ground-up filter paper.

The general tendency in describing cellulose organisms has been to regard the cellulose-decomposing ability as a characteristic outweighing others in importance, and therefore to suggest that new genera be established as a recognition of this fact. At various times the following genera have been set up: *Cellulomonas*, *Cellvibrio*, *Cellfalcicula*, *Cytophaga*, *Cellulococcus*, *Cellulobacillus*. The first four of these appear in the most recent edition of Bergey's Manual, but even so are questionably defensible, on one ground or another.

In 1923 Bergey (39) suggested a tribe *Cellulomonadaceae* of the family *Bacteriaceae* (Conn.) which grouped together all the short rods isolated by Kellerman *et al.* (32, 38), that had the property of digesting cellulose. This tribe was represented by a single genus *Cellulomonas*, of short, non-spore-forming rods with rounded ends, occurring in the soil. An additional characteristic was "Growth on ordinary culture media often not vigorous." Other cellulose decomposing rods were included in the genus *Bacillus*, the morphology alone being used in classification. This precedent was followed as other organisms were identified even though they did not fit too satisfactorily into the genera to which they were assigned (42, 44).

Winogradsky (9) proposed three new genera, *Cytophaga*, *Cellvibrio* and *Cellfalcicula*. In his own words. . . "le nom *Cytophaga* paraît bien expressif; pour les autres, on pourrait garder les anciens noms se rapportant à la forme, mais en ajoutant l'abréviation *Cell*; on dirait ainsi *Cellvibrio* au lieu de *Vibrio*, en précisant par ce nom générique la forme et la fonction." The genus *Cellfalcicula* of which three species were described was entirely new and consisted of short, non-spore-forming rods with pointed ends growing feebly on "ordinary culture media." Apart from the distinction between round and pointed ends there appears to be little difference between the species described and species of *Cellulomonas*. The genera *Cellvibrio* and *Cellfalcicula* were keyed under the family *Pseudomonadaceae* and tribe *Spirilleae* in Bergey's Manual.

The prefix *Cell-* is presumably acceptable as meaning cellulose, although not in accordance with the precedent of *Cellulomonas*, in which *Cellulo-* is used in the same sense. The brevity of *Cell-* in combination with morphological terms has something to recommend it, although in its present use it does not have a Latin root, since both "cella" and "cellula" have other meanings. For absolute clarity perhaps *Cellulo-* is preferable, but there are combinations, *e. g.*, *Cellulovibrio* in which this would be less euphonious.

One of the characteristics specified for both *Cellvibrio* and *Cellfalcicula* is that they "oxidize cellulose, forming oxycellulose," and that "growth on ordinary culture media is feeble." The former statement is not believed to be accurate as indicated on page 248, and the latter, because of its vagueness, may cause confusion. As additional organisms are isolated and their growth requirements better understood exceptions would almost certainly have to be made or else new genera set up differing only in the vigor with which non-cellulosic carbohydrates are attacked. On several counts, then, it seems that some revision of these two genera would be desirable.

The systematic position of the cytophagas has recently been placed on a much more satisfactory basis (36, 43). Hutchinson and Clayton (42) originally placed their interesting organism in the order *Spirochaetales*, recognizing at the time that this was not wholly satisfactory, and subsequently it has been included under *Actinomycetales* (51) and *Myxobacteriales* (36). The classification of the cytophagas offered by Winogradsky (9) included in Bergey's Manual (40) placed them in an appendix to the order *Spirochaetales* under the family *Spirochaetaceae*. The statement is made "Because the relationships of these cellulose-destroying organisms to organisms in the genus *Spirochaeta* are not clear, all the species that have been described as belonging to the genus cytophaga have been placed in the appendix." Stanier (43) points out that the peculiar characteristics of the vegetative cells of the cytophagas, the presence of flexing movements, the mode of locomotion, etc., are sufficient to exclude them from the true bacteria. He includes them in the order *Myxobacteriales* under two families, *Myxococcaceae* and *Cytophagaceae*, differing only that in the latter "sporoids" or microcysts are absent. The genus *Sporocytophaga* under the former would have as type species the original Hutchinson and Clayton organism, and the genus *Cytophaga* under the latter would have as type species *Cytophaga hutchinsoni* Winogradsky. It would be unwise to write into the generic descriptions the requirement that cellulose shall be utilized or that cellulose only shall be utilized. The separation at present

is entirely on morphological grounds and will be less likely to be upset if it remains so.*

In general it would seem that the creation of new genera on the basis of cellulose-decomposing ability alone should be approached with more caution than has been the case, particularly when the general fermentative characteristics of the organisms, apart from the ability to utilize cellulose, do not differ appreciably from others similar morphologically.

5. *Fungi*

It was early recognized that fungi play a significant role in the decomposition of cellulose, but specific investigations with fungi have curiously enough lagged far behind those with bacteria. In all attempts to isolate cellulose bacteria from the soil by direct inoculation of filter paper with soil, abundant growth of fungi is often obtained. The incorporation of cellulose into the soil, particularly if ample available nitrogen is present, is always followed by a heavy development of fungal mycelia. McBeth and Scales (52) were able to demonstrate the growth of many fungi on regenerated cellulose agar plates. Scales (53) studied the cellulose-decomposing ability of 30 species of *Penicillium* and 10 species of *Aspergillus*, the growth of which on plates was usually accompanied by the enzymic production of a cleared zone round the colonies. The extent of growth of fungi on such plates, however, is not a good indication of the vigor with which either isolated cellulose or the cellulose of plant materials may be attacked. Many of the Basidiomycetes, for example, cannot be satisfactorily grown on plates and yet may be shown by analysis to be capable of accomplishing extensive cellulose decomposition. There is some evidence that, in soil, fungi may be more vigorous than bacteria in the decomposition of cellulose. Jensen (54) studied the relative decomposition of filter paper and straw cellulose in sand inoculated with pure cultures of some fungi and bacteria, and in general the fungi brought about the more extensive loss in equal time.

The list of fungal species capable either of growing on pure cellulose or attacking the cellulose of plant materials is a very long one and no useful purpose would be served by listing them. However, it is not to be as-

* In a recent communication by Stanier [*J. Bact.*, 42, 527 (1941)] two new species of *Cytophaga* of marine origin were described, which attack cellulose slowly, but the other biochemical characteristics of which are completely at variance with the physiological requirements of the genus as described by Winogradsky. They are by no means specialized, but utilize, somewhat feebly, a wide range of compounds. It is clear that only the morphological characteristics of this genus should be retained.

sumed that all fungi have this ability though the majority unquestionably do so. Among the common fungi, species of *Mucor* (55, 57), *Rhizopus* (56) and *Oidium* (57) seem to be unable to develop on cellulose.

Fungal damage of cotton, cotton goods, textiles and paper is a serious problem, particularly in storage. Cellulose fibers are markedly hygroscopic and some fungi possess the power of developing at relatively low moisture levels. Growth is usually very restricted partly for this reason and partly because the nitrogen level may be low unless some form of size which contains nitrogen has been used. The damage ordinarily caused is not dramatic in the sense that holes are produced in the fabric or paper, but is usually marked by a deterioration in strength, and staining due to the color of the fungal growth.

6. *Actinomycetes*

Although the widespread distribution of actinomycetes in soil, composts and other places where plant materials are decomposing aerobically has long been recognized, extremely few studies have been made of their physiology, particularly with respect to cellulose utilization. Ordinarily they grow rather slowly, and at relatively low moisture levels. If soil is plated out in one of the cellulose agar media, actinomycete colonies are rarely seen, but this may be because the plates often are overgrown by fungi before the actinomycete develop. Actinomycetes can, however, be grown on cellulose plates or on filter paper if the moisture conditions are favorable. Scales (53) studied the cellulose-decomposing ability of 31 cultures of actinomycetes and found that eight caused obvious digestion of the cellulose in cellulose-agar plates. Waksman (58), in developing a provisional key for the classification of this group, demonstrated that many species can utilize cellulose, and seemed inclined to the view that this might be a general property common to all.

V. Decomposition of Cellulosic Materials

In any plant material that is extensively decomposed under natural conditions, either aerobically or anaerobically, the major constituent lost is invariably cellulose. Soil organic matter, which represents the residues of past vegetation, contains practically no cellulose, and similarly cellulose can rarely be detected in lake or river mud deposits. Most natural decompositions involving cellulosic materials are accomplished by mixed floras, and the transformations that occur are not easily related to pure cultural studies on isolated cellulose. It is not possible to formulate any opinion as to the importance of a particular organism in a mixed culture,

even though its behavior alone and in comparison with similar organisms is known. For example, much attention has been given to *Cytophaga* in pure culture, but this provides no hint as to the part which this organism plays in the decomposition of cornstalks in soil. There is some doubt, indeed, whether the highly specialized cellulose organisms are, in fact, of any real importance in the utilization of the cellulose of plant materials under natural conditions, inasmuch as more versatile forms would be favored. Natural decompositions in a sense must be highly competitive with respect to any one constituent, since only those organisms the activities of which dovetail together so that the available energy is utilized most efficiently will prevail. Sequential changes in the population, therefore, are an inevitable result of substrate heterogeneity. Furthermore, it is highly improbable that any one organism in a mixed flora would be responsible for the removal of all the cellulose that is lost from a plant material that has undergone substantial decomposition. Indeed single organisms do not seem able to accomplish either so rapid or so extensive removal of cellulose from plant materials as mixed populations. Comparisons of the activity of certain fungi on oat straw showed that the most active of those studied, *Aspergillus versicolor*, brought about in 48 days a loss in weight of 29.3 per cent, of which the major part was cellulose, whereas 38.9 per cent was removed by a mixed culture under the same conditions and in the same time (59). Similar results were obtained on wheat straw and oak leaves with a variety of organisms (61), on oat straw, cornstalks and alfalfa (56), and on composts of straw with alfalfa at temperatures in the thermophilic range (61).

There is some reason for believing that the association of organisms of entirely different metabolic character, such as bacteria and fungi, are especially effective. Waksman (56) has shown that the qualitative and quantitative activities of one organism may be substantially changed by the presence of another. In the decomposition of alfalfa, *Trichoderma*, ordinarily a vigorous cellulose organism, attacked mainly the proteins in pure cultures, but in the presence of *Rhizopus* which can utilize proteins but has not the ability to attack cellulose, *Trichoderma* developed at the expense of the cellulose. Even the nature of decomposition produced by a mixed population was modified by a brief preliminary attack by a pure culture.

Because of the structural characteristics of the cell-wall, the effect of the presence of one constituent on the availability of another is a matter of considerable importance in natural decompositions. Particularly is this the case with respect to the effect of lignin on the utilization of cellulose.

Lignin, while not wholly unavailable, is the most resistant of the major plant constituents, either aerobically or anaerobically, and its distribution encrusting and interpenetrating the cellulosic fabric therefore has a considerable influence on the readiness with which the cellulose itself may be utilized. It is a matter of common observation that plant materials are less rapidly and extensively decomposed as the extent of lignification increases. Since lignification is a process of maturity, there are concurrent changes in composition which must also be taken into account, such as a reduction in protein content and in water-soluble constituents. When due allowance is made for these, however, it is found that the cellulose of a material containing 5 per cent lignin is far more easily attacked than the cellulose of a material containing 15 per cent. The lignin content of most woods falls within the range of 20–30 per cent and it is found that the cellulose of woods is not readily attacked by the same type of mixed flora that will effect substantial decomposition of straw, even though wood shavings or sawdust be used, ample nitrogen added and alcohol-soluble resins, that might be inhibiting, removed. Coir fiber with a lignin content of over 30 per cent is one of the most resistant plant materials and maintains its structure even if buried in the soil for long periods. Slow decomposition of the cellulose of intact wood and timbers is accomplished by many Basidiomycetes, and it may be that attack by such organisms is less hampered by the presence of lignin than ordinary aerobic or anaerobic floras. Rege (62) developed the thesis that lignin might be considered to be an “inhibitory” factor in contrast to the “pentosans” which he regarded as an “energy” factor, and that the ratio of the latter to the former might be used to predict decomposability. While this left out of account the main constituent attacked in any normal decomposition, namely, the cellulose, it involved the recognition that increasing lignin content is usually accompanied by an increasing resistance to decomposition. It is curious that the presence of a small percentage of lignin adversely affects the availability of isolated cellulose to a greater extent than in whole plant tissues. The presence of more than 1 per cent lignin was shown substantially to reduce the extent of fermentation of pulps by a mixed culture of thermophilic anaerobes. The action of the lignin seemed to be passive, not active (15).

The presence in plant materials of carbohydrates more widely available than cellulose but permitting the rapid development of a vigorous flora seems to pave the way for the attack on cellulose, the removal of which in equal time is substantially slower if the soluble constituents and hemicelluloses are first removed (63). In a comparison of the aerobic decomposition of oat straw and acid-extracted straw, 73.8 per cent of the former

was lost in ten months and only 29.4 per cent from the latter. While this comparison is not entirely valid because in the process of acid extraction the cellulosan fraction would be largely removed from the oat-cellulose, along with more available constituents, the difference is very great.

The facts presented in the preceding three paragraphs taken together make it certain that there are important aspects of the decomposition of cellulose in natural processes which are incompletely explained by pure cultural and pure substrate studies. Three ramifying lines of investigation must be pursued. First, the activities of the cellulose-decomposing organisms on plant materials in pure culture must be ascertained, since there is reason to believe that the availability of plant cellulose associated as it is with other constituents may be of a different order from that of filter paper strips. Second, the effect of composite cultures in accomplishing more extensive or more rapid decomposition of plant materials than single cultures must be examined. Third, the implications of varying composition and structure must be studied with both pure and mixed cultures.

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A UNIFIED HYPOTHESIS OF THE RECIPROCAL INTEGRATION OF CARBOHYDRATE AND FAT CATABOLISM

By

EDGAR J. WITZEMANN

Madison, Wis.

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The fact that fat and carbohydrate catabolism are integrated is one of the most securely known facts in the physiology of metabolism. The manner in which this integration is obtained is, however, one of the most obscure aspects of this physiology. It is the purpose of this essay to provide for such an integration, and in doing so to take full account of the best known facts relating to both types of substances. It seems advisable to enumerate these facts, along with such comment as seems desirable, before attempting such an integration, so that they may not be confused with what we shall say about them. They are presented in this brief form as familiar for the most part, and are not believed to require full documentation. Many other familiar facts and especially supporting and suggestive details are omitted, because they are related to, or covered by, others here listed. Since the picture to be presented is a tentative or working hypothesis, which is to be drawn in broad lines lacking detail, the omission of such detail is thought to be justified.

For the sake of brevity and convenience the facts enumerated will be presented in numbered paragraphs.

I. Fundamental Facts Relating to Fat and Carbohydrate Catabolism

1. It has been known, ever since men first began to observe themselves and each other, that other things being equal, starchy foods, that is carbohydrates, contribute to maintain the body weight, and especially to maintain the fat in the fat depots, chiefly observable in the skin. Likewise it was realized that abstention from food, or a restricted diet, leads to a loss of weight, chiefly due to the loss of fat from the fat depots, at first. Through the centuries, and especially in the last century, the data have been refined and perfected until it is impossible to doubt the over-all statement that fat and carbohydrate catabolism are integrated in this way.

2. How great the reserve of fats is may be indicated by the fact that of a 140-pound man about 90 pounds is water, and of the remaining 50 pounds, 14 pounds consist of fatty substances, and only 1.4 pounds of carbohydrates. Thus statistically considered, fats are ten times as important as carbohydrates in the constitution of an average man. The picture is completed with 28 pounds of protein and 7 pounds of minerals. Thus chemical analysis would support the idea that in starvation fats might be expected to be utilized for maintenance, and the fact that such reserves are largely laid down on the exterior of the body makes their diminution promptly evident.

3. The converse picture is also interesting. Although the fat reserves can be increased beyond all needs, as in a hog, for instance, the carbohydrate reserves can only be materially increased temporarily, since they are slowly converted into fat, if present in excess of a certain optimum. Thus a hog may dependably be used to convert low-grade carbohydrate into high-grade fat.

4. As a whole, the chemistry of neutral fats is the simplest of that of any abundant substance in the animal organism, except that of water. The most abundantly found constituent fatty acids, of fats and oils, contain eighteen carbon atoms, *i. e.*, stearic and oleic acids. The remaining naturally occurring fatty acids consist almost exclusively of fatty acids containing an even number of carbon atoms, and of these palmitic acid is probably the most abundant. Fatty acids with twelve and fourteen carbon atoms occur more abundantly in plants than in animals.

5. This absence of the intermediate acids with an odd number of carbon atoms led to the assumption that the carbon chains of fatty acids are built up and broken down, two carbon atoms at a time. That this holds true of their synthesis has never been proved experimentally, and the overwhelming abundance of the eighteen carbon atom acids, which occur in

considerable variety, suggests that the most frequently used unit in the synthesis may be a six-carbon chain, *i. e.*, a hexose unit. Considering the ease with which glucose when stored as glycogen is converted into fat in the animal organism, this suggestion is not irrational (22) although little more than an over-all picture is available. Perhaps the clearest picture of this change is obtained in the conversion of starch into fat in ripening olives and castor beans, which has been much investigated in certain directions (9).

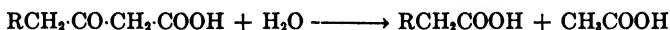
6. On the contrary, that fatty acids are broken down in physiological oxidation two carbon atoms at a time, or possibly sometimes four at a time, is scarcely questionable, on the basis of existing evidence. Perhaps the most direct evidence is the fact that the acetone "bodies" and acetic acid (17) make their appearance in the urine and the body fluids, when carbohydrate oxidation is deficient. Careful studies have shown that these are derived from fatty acids, with the exception of a smaller amount derived from the breakdown of leucine, tyrosine and phenylalanine.

7. This conception was clarified by the pioneering work of Knoop (11), who prepared ω -phenyl substituted fatty acids, and subjected them to oxidation in the animal body. The acids with an even number of carbon atoms in the side-chain gave rise to phenyl acetic acid, which was in part excreted as a conjugation product in the urine. Those fatty acids with an odd number of carbon atoms in the side-chain gave rise to benzoic acid. Moreover, there was reason to believe that this was accomplished by the so-called β -oxidation, especially in view of the fact that the administration of the β -oxidized parent substance gave rise to the same stable end-product, as when the unoxidized parent substance was administered.

8. According to available information in organic chemistry forty years ago (4), there was no reason to believe that the oxidation of a fatty acid could be inaugurated on the β -carbon atom as suggested above, or that a fatty acid so oxidized could lose two carbon atoms at one time, or in one piece. Dakin, however, found that the β -carbon atom is attacked if the ammonium salt of the fatty acid is treated with hydrogen peroxide in aqueous solution. Since then the writer (23) has found that other catalysts act in the same way, and that both the α - and the β -carbon atoms are then attacked simultaneously, especially when butyric acid is used, as was also observed by Dakin. The writer has also established that under suitable conditions, *i. e.*, in alkaline solution α -hydroxy fatty acids can be caused to lose one or two carbon atoms. When two are lost in this way they can be recovered in one piece (as oxalic acid (24)), which has never been accomplished for the β -oxidation.

9. Other workers, especially Embden and his associate (5) soon confirmed the implications of the above paragraphs by perfusing freshly excised liver with salts of fatty acids. They found that beginning with butyric, the acids with an even number of carbon atoms gave rise to the formation of acetoacetic acid, while those with an odd number of carbon atoms failed to do so.

10. In all of this earlier work it was implied, if not stated, that the β -keto intermediate acids believed to be formed undergo a hydrolytic decomposition, which could be represented thus:



Such reactions are known to occur *in vitro*, and with acetoacetic acid the reaction requires high alkali concentrations for its accomplishment. Heretofore all of the many attempts to demonstrate the existence of this reaction in the organism, and in conditions other than in these strongly alkaline solutions have failed. Of course it has been suggested that the two carbon fragment is oxidized in the organism, before or in the act of being dropped, and could therefore not be recovered as acetic acid. Thus in this form the theory provides for a repeated β -oxidation of the even-numbered fatty acid so obtained until it is converted into acetoacetic acid.

11. Aside from being unproved, this theory raises a rather pertinent question as to the intermediate short-chain fatty acids that would be formed in such a process. So far these have not been encountered. To anyone familiar with the odor of butyric acid in traces, as it is encountered in regurgitated food and vomit, where it is so readily and unmistakably identified by its odor, it seems that olfactory proof, at least, for the presence of this substance would have been obtained in many situations, if this acid is really formed in such oxidations (10). So far even such qualitative evidence is lacking. The great solubility of butyric acid in aqueous solutions, in contrast to the limited solubility of higher fatty acids, would favor its desorption from enzyme systems, and increases the significance of this negative evidence.

12. In these earlier studies the existence of other points of primary attack could be excluded on the basis of the lack of evidence, and because the existing data offered what appeared to be a clearer and simpler explanation of the available facts than any alternative hypothesis with even less experimental support, would give. Thus even the recently discovered ω -oxidation of fatty acids has been shown by its discoverer to be, at most, of minor significance in fat catabolism.

13. Twenty years ago, before insulin was available, the otherwise smoothly working over-all integration of carbohydrate and fat catabolism of normal individuals became an acute problem when in severe diabetes the increasingly deficient carbohydrate catabolism presently became associated with ketosis. This relationship was so clearly recognized already in 1905 that it could be expressed aphoristically: "the fats burn (completely) only in the fire of the carbohydrates." In these desperately ill people it was established that when the molecular ratio of fatty acids being burned, to glucose units also being burned at the same time, exceeded a certain ratio (*i. e.*, 2:1 for the over-all catabolism) ketosis was observed. This ratio is probably not a fixed ratio for all conditions involving ketosis, but indicates that at some crucial point some aspects of the two catabolisms are linked. These facts repeatedly observed in such situations demonstrate that these two catabolisms are somehow linked or coupled, and this fact cannot be set aside, as has been proposed by some workers (19), because the ratio mentioned is not constant under all circumstances. Nor can it be assumed that the degree and manner of linkage must be the same in all circumstances in which it may be expected to exist.

14. Thus it appears that in living cells, fats or fatty acids are easily burned at least partially, and, in fact, in some conditions, as in diabetes, they are more easily burned to this extent than carbohydrates. Thus from the standpoint of the quantity available, and the readiness of oxidation, fats are indeed the fuel reserve *par excellence*, by common agreement. However, it is also agreed that in the test tube, fatty acids, their salts, as well as fats themselves, are usually quite difficult to burn in aqueous media, even with strong oxidizing agents. However, with hydrogen peroxide, in a mixture of phosphates, butyric acid is more rapidly burned than glucose (25). Under these conditions two molecules of butyric acid are destroyed before all of one molecule of glucose is destroyed, when both are present in the solution at the same time. The writer has never ceased to be amazed by this result, so deeply is the traditionalized experience with these two types of molecules imbedded in our chemical attitudes. Both the physiological facts cited above and these *in vitro* observations should serve to prepare us to see fatty derivatives burning in the presence of carbohydrate derivatives.

15. Another point that is obvious is that because the formation of fat from carbohydrate, and its oxidative catabolism, are so strangely and completely reciprocally linked to the oxidative catabolism of carbohydrates, the two are not mediated through the same chemical and physico-chemical circumstances. Such a separation would, however, exist only in the initial

stages of the processes. Moreover, it is equally evident that they do become integrated at some later stage, or else fat catabolism could not have the "pinch-hitting" relationship to that of carbohydrate that it has, and one of the difficulties of this problem is to provide for both a separation and an integration.

16. Perhaps the solubility relations of fats are the most fundamental facts to be considered. Neutral fats are among the most insoluble substances existing in living organisms. Moreover, in man major portions of neutral fat are laid down in the skin, and in other fat depots, in which oxidative catabolism of these fats would, even if it occurred there, be of little value to the remaining tissues. Thus it is almost inconceivable that these depot fats could be effectively oxidized for the metabolism of maintenance *in situ*. How can they be moved elsewhere since they are insoluble?

17. Fat hydrolyzing enzymes, by which the neutral fats could be converted into the free fatty acids, are widely if not universally distributed. The fatty acids so formed would form the soluble alkali soaps in favorable conditions. In man the pH values in tissues and body fluids are barely favorable to the existence of such soaps. Moreover, the higher fatty acids themselves are quite insoluble, and so without the aid of additional factors, it is inconvenient to think of fats being mobilized and burned in the tissues mainly as soaps.

18. On the other hand the peculiar anomalous half-soluble fats called phospholipids occur widely and have long been suspected of playing important roles in the organism. The glycerolphosphoric-choline part of these molecules is water soluble, while the fatty acid radicals are not. Thus these substances constitute a type of bridge across the gap of water solubility and insolubility. Because of this, lecithin, although insoluble in water, is spontaneously peptized, *i. e.*, dispersed in water, and is thus an example of one of a few types of water-insoluble substances that spontaneously adopt the state of colloidal dispersion in water (16). As such it could and does exist in the blood, for instance.

19. It has also been known, for some thirty or more years, that the bile salts of the bile exercise a solubilizing effect on higher fatty acids, by which they enter into a complex, which has the amazing property of being diffusible and dialyzable in aqueous systems (21). This property is known to be especially pronounced with desoxycholic acid. Since the bile salts in being absorbed from the intestinal tract perform this action with fatty acids derived from the diet, and thus come into the blood in the so-called "circulation of the bile," and since other physiologically important substances, such as cholesterol and its esters, are fundamentally similarly constituted, it

raises the question whether these substances can also play a role in the solubilization of the neutral fats in the depots, especially after its digestion by lipase, and in their transport and utilization in the tissues (2).

20. It is assumed that the widely accepted picture of carbohydrate catabolism initiated by Embden (6), so far established especially for *in vitro* conditions, is correct in principle, and applicable, in its essentials, to the conditions existing in tissues, both in its anaerobic form and in some aerobic form. Moreover, it is assumed that this catabolism is "tuned" in such a way that in normal conditions, that is, in the presence of adequate supplies of carbohydrate, the catabolism of these furnishes the major part of the energy needs of the organism, even in the presence of an abundance of fatty derivatives (*cf.* items Nos. 1, 2 and 3 above). It is likewise regarded as clear that this selective "tuning" is mainly obtained by the remarkable properties of the phosphagen catalytic system and the glucose phosphoric esters and derivatives formed by it.

II. The Mobilization and Catabolism of Depot Fats

Anyone who has considered this problem has realized that the mobilization of depot fats, and their oxidative catabolism, must be visualized as parts of a continuous process analogous to the prevailing conceptions of the mobilization and catabolism of carbohydrate.

Recently it became evident to the writer that by utilizing the facts presented under items Nos. 16, 17, 18 and 19, especially the last two, a tentative hypothesis that takes account of the remaining facts, and has the quality of continuity, can be constructed.

As we have stated the phospholipids are really constituted of two parts: the glycerolphosphocholine radical, and the two attached fatty acid molecules. This glycerolphosphocholine radical, in a gross way, reminds one of the nucleotides that function as prosthetic groups in some of the enzymes involved in carbohydrate physiology, and the suggestion arises as to whether this radical can function in some such fashion in fat catabolism. Similarly the phospholipids may be regarded as analogous to the monophosphoric ester of glucose, which in itself is inert and which becomes active only when it has become attached to the enzyme system by which transphosphorylation, molecular rearrangement and further phosphorylation are brought about, and by which it is made ready for the succeeding events of this cycle. Here the enzyme system, as is apparently usually true for the enzymes of tissue respiration, is the fixed component, and the organic substrate and the oxygen are brought to it in a relatively inert form.

Thus in a familiar instance we have liver glycogen, that is, depot glycogen, mobilized and transported in the blood as inert glucose, which is again converted into glycogen in muscle. Here it is again mobilized as a comparatively inert phosphate ester on the if, as, and when basis that normally controls all metabolic processes. In an analogous way we may now see the depot fat mobilized as phospholipids, or as the cholesterol-cholic acid system. It is transported in this essentially inert form, and brought to the tissue enzyme systems, where, on a parallel but not identical if, as, and when basis, it may be considered to undergo changes analogous to those taking place with the glycogen unit that lead to its activation and eventual combustion. In this connection it is to be recalled that Leathes (13) and Bloor (1, 2) have in the past emphasized such a role for these hydrophilic fatty compounds, and it is by this parallel projection of the glycogen and fat systems that these suggestions begin to become obvious.

If then we allow the phospholipid to be taken up by the enzyme system, since we must start with one type of substance, we would first expect to see it acted upon by a lecithinase (or lipase), an ester linkage hydrolyzed, and a fatty acid molecule formed.* The water-insoluble hydrocarbon part of the fatty acid molecule will, by this time, have been oriented and perhaps adsorbed. Likewise by a slight change in position it could react through its newly liberated carboxyl group to form a salt with the basic choline radical. The fatty acids are fairly strong and choline is a strong base, so that a substituted choline salt of the fatty acid could be formed, if the other circumstances are favorable. Thus through some or all of these circumstances the fatty acid comes into such a position in the enzyme system that it is susceptible to oxidation, that is, dehydrogenation and conversion eventually into carbon dioxide, by mechanisms analogous to, or identical with, those functioning in the oxidative dehydrogenation and decarboxylation of carbohydrate. Such a fatty acid molecule would furnish hydrogen if, as, and when it is needed in the other processes of the cell. When finally destroyed in this manner, the first fatty acid molecule could be replaced by the remaining fatty acid molecule, and the glycerolphosphocholine would eventually be free to enter the cycle again, or itself undergo oxidation. The events taking place after the destruction of the first fatty acid molecule could be visualized in several ways. One interesting possibility would be

* The question as to whether a fatty acid in ester combination, as in a fat, for instance, is attacked oxidatively before the ester linkage is hydrolyzed has perhaps never been investigated. In the absence of data we will make the traditional assumption, that it is first hydrolyzed and then attacked. This is also supported by the widespread occurrence of lipolytic enzymes.

that the free fatty acids and soaps of item No. 16 are sometimes taken up at this point by the glycerolphosphocholine, which for the moment constitutes a part of the enzyme system, and are thus brought into the zone of action, although by themselves they are, perhaps, inert and incapable of entering this zone.

In this way we have made use of the primary properties, and the individual components of the phospholipids, in the above suggestions, and have assigned another functional meaning to the almost universal distribution of phospholipids and lipase in the organism. But more especially, we have provided a vital role for choline, which is known to play an essential part in fat catabolism. Moreover, because basically this suggestion rests on the transformation of fat into hydrophilic substances capable of transport to the tissues, we can by extension include the cholesterol and cholic acid derived systems that likewise are known to be significant in the transport of fatty acids in similar hydrophilic systems. Thus we may use these substances to set up an alternative type of hypothesis, the fundamental mechanics of which would be the same, but which by assumption differs in some essential points from the other hypothesis. This is done because, as we shall point out later, we wish to have another simultaneous mode of fat catabolism. Our hesitancy in using these compounds for the physical job of fat transport, in physiological oxidation, arises from the fact that it may be that the phenanthrene derivative involved is sometimes being transported by the aid of the fatty acid; that is, these substances like the phospholipids, and their components, have other properties and other functional roles in life besides the one that we have chosen to emphasize. Be that as it may, they are known to be indispensably involved in the lowly task of fatty acid absorption in the intestine, and the task in which we have assigned them a role may be regarded as one of a higher order.

Having in the above provided ourselves with two primary mechanisms for fat catabolism, we may assume that one of them is the predominant process by which fat is burned normally and simultaneously with carbohydrate, without the complicating circumstance of a significant production of acetone bodies. For this mechanism we may choose the phospholipid as the fatty acid carrier. In this case the provision of any intimate integration between the two catabolisms is not required, beyond the fact that the glycerolphosphate may be derived from carbohydrate, in part at least.

Now if and when carbohydrate is not, or cannot, be utilized the energy needs of the organism would need to be met more completely from fat (*cf.* items Nos. 1, 3, 13 and 20), and the above mechanism would then be called upon to a maximal extent. However, under these conditions the acetone

bodies tend to make their appearance in the blood and urine. It would in this situation be simple to assume that they are formed by another mechanism, which normally plays a relatively insignificant role in the energy metabolism as a whole. Thus following a suggestion made by Mathews (15) desoxycholic acid, or its conjugated or related cholesterol derivatives, could adsorb the carboxyl end of the fatty acid radical in such a way that the α -carbon atom would be inaccessible to dehydrogenation effects, at first at least, and the β -carbon atom would become oxidized. In this instance the fatty acid might be considered to yield acetic acid, and especially acetoacetic acid, and this would account for the appearance of these anomalous compounds under such circumstances (*cf.* items Nos. 10 and 13). Of course here again the picture could be set up in a variety of ways, but the physical fact of the solubilizing action of this bile acid on insoluble fatty acids has been made use of as an essential factor at an early stage of the process. The differences in the orientation of the fatty acid molecule in these two sets of circumstances are regarded as the underlying causes of the differences in the breakdown of the fatty acid molecules. Moreover, it is implied that this second reaction mechanism is normally not very active in the presence of metabolizing carbohydrate, so that the disposal of the end-products of this process is never normally a serious problem, quantitatively considered.

Moreover, in general it is assumed that in the first process the reaction mechanism follows some form of an α -oxidation, and its possible sequences, in which the fatty acid would perhaps tend to become desorbed only when it had been converted into the extremely soluble acetic acid. In the second process this desorption could also occur with acetic acid, but more especially with acetoacetic acid. Here, in order to complete the oxidation of these kinetically active molecules, we may provide a corollary hypothesis in the nature of a "recapture synthesis," by which these molecules, if and when they escaped from the primary enzyme system, would be taken up by some phase of the carbohydrate system, and metabolized in this system. Such a recapture synthesis may at first sight appear to be a novel chemical device, but this is not true.

Such recapture syntheses represent a principle widely used in the organism. In digestion the absorbed material is largely converted into diffusible, kinetically active, molecularly disperse molecules, which are recaptured for growth, repair, the deposition of glycogen and the like. The resynthesis of glycogen from lactic acid is also such a synthesis, in which an inert molecule is brought back into a useful form. Such syntheses are also known *in vitro*. Thus Shaffer, *et al.* (18), long ago brought acetoacetic acid to

react with sugar-breakdown intermediates, and Henze, *et al.* (7), more recently, found that it reacts with such products under mild conditions, to form labile reaction products. A recapture synthesis of this sort that is much discussed is represented by the so-called citric acid cycle, especially when this is viewed somewhat broadly. It is even possible that the same enzyme system is functioning in many instances involving such carbon to carbon condensations.

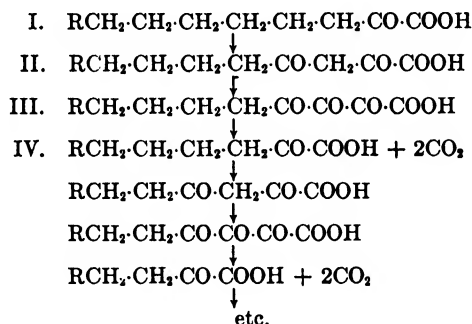
The net effect of such a recapture would be that these kinetically active molecules will be taken up and disposed of by oxidative dehydrogenation as long as the circumstances for the synthesis are favorable, and these compounds, *i. e.*, acetic acid and acetoacetic acid will only be found in the urine if and when this synthesis proves to be inadequate, *i. e.*, in the circumstances existing in ketosis, for instance.

Summarizing we may say that the above suggestions concerning fat catabolism consist of two parts. First the fats are rendered soluble enough for transport in the form of two types of hydrophilic systems. In this form they are thought to be conveyed to the enzyme systems, where, because of differences in their transport on the carrier systems, their oxidative breakdown is inaugurated in two different ways. It is further provided that both of these mechanisms may give rise to products that must be disposed of in some other way, if the fatty acids are to be completely oxidized. This is thought to be accomplished by a secondary or recapture synthesis. Furthermore, it is considered that it is a change in the quantitative significance of these three processes that makes the shift from an essentially pure carbohydrate catabolism to an essentially pure fat catabolism possible, when this is required for the life of the organism.

Thus the phospholipids and the cholesterol-cholic acid-fatty acid systems would constitute the prosthetic groups of the enzyme systems by which the fatty acids, which they transport, are brought to the site of their catabolism. The remainder of this enzyme system would be structurally fixed in the cells. Moreover, the enzyme systems here visualized differ in some fundamental respects from those visualized for carbohydrate, as indeed they must in order to be linked in the peculiar reciprocal way that they are, to the activities of these enzymes. The former must and do function when the latter fail, and for this they would need to be fundamentally different at some essential points in their mechanism and action.

The α - and β -Oxidation of Fatty Acids.—In this unified hypothesis it is assumed that both primary α - and β -oxidations are involved in the oxidative breakdown of fatty acids. Both were shown to exist in Dakin's studies of the oxidation of butyric acid, and the writer has repeatedly con-

firmed and extended these observations. The traditional argument that α -oxidation cannot be involved in physiological conditions, because it leads to the loss of one carbon atom from the chain with the formation of unnatural fatty acids was based on erroneous and incomplete information. Thus as it now stands both mechanisms may lead to the loss of either one or two carbon atoms, depending on circumstances. For the α -oxidized fatty acids there is a general tendency for a three-carbon acid to lose one carbon atom, but this tendency is diminished, and almost abolished so that two carbon atoms are lost, and may even be recovered in one piece, as the alkalinity of the solutions, or the length of the fatty chain, is increased (25). Thus both mechanisms are required to account for the results of *in vitro* oxidations, and both should be retained for physiological conditions until one or the other is shown not to exist. Such a retention is also convenient because the α -oxidation avoids the formation of the acetone bodies. On the other hand, the β -oxidation conception provides for their formation, when they appear. Both conceptions can be developed in a way that provides for the non-formation of the short-chain acids, which are not found (*cf.* item No. 11), and for the formation of which the more traditional conceptions so carefully provided (*cf.* items Nos. 10 and 11). Thus α -oxidation can be visualized as taking place thus:



In the scheme of α -oxidation it is assumed that after oxidation of the α -carbon atom the γ -carbon atom is attacked. This mechanism is purely conjectural, so far as the attack on alternate carbon atoms is concerned, but would thus be analogous to a much considered form of the β -oxidation conception, in which the idea of an alternate oxidation seems to be required. The limited multiple alternate α -keto acids so provided for would be extraordinarily active, and could be expected to undergo various secondary reactions involving possibly the formation of ring systems like cholesterol, and

its derivatives, and perhaps at times the formation of carbohydrate, *i. e.*, glycogen. The mechanism as given is also based in part on the notion of avoiding the formation of normal fatty acid intermediates, and on the behavior of acetopyruvic acid and acetyl acetone when oxidized (unpublished data). The α -keto acids visualized as intermediates would be more "active" than the corresponding unsubstituted fatty acids, and would thus provide for the continuation of the oxidation of a given fatty acid molecule. Thus it is also assumed that the oxidation of alternate carbon atoms advances along the chain just a little ahead of the dropping-off of carbon atoms. The detailed mechanism of the loss of two carbon atoms actually or virtually at the same time is unknown, but it is not assumed that oxalic acid is necessarily an intermediate in this process. In *in vitro* oxidation in solutions sufficiently alkaline to allow of the formation of a normal oxalate, oxalic acid is recovered as a stable end-product and thus gives proof that the two carbon atoms are, in such conditions, lost in one piece, and at one time, but no evidence is available to indicate that it is formed by simple hydrolysis of such a triketo acid as (III) above, although this could be expected to occur. Moreover, the evidence is clear that such triketo acids can exist as intermediates in the *in vitro* oxidations. Such derived intermediates as (IV) would be so unstable that they would serve to provide for the continuation of the oxidation of the fatty acid molecule in process of oxidation, and thereby prevent the attack on more resistant unattacked molecules that have not yet become involved in the oxidative process. The formation of such an intermediate would therefore serve to control the course of chemical events in the catabolism of fatty acids and provide for a continuation of the oxidation of the reacting molecule.

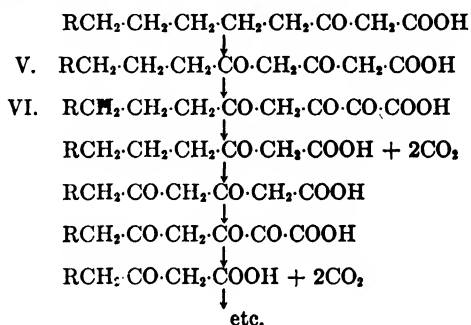
It is not considered that when (II) is oxidized (III) is invariably obtained. In fact it is to be expected that the triketo acid, $\text{RCH}_2\text{-CH}_2\text{CH}_2\text{CO}\cdot\text{CO}\cdot\text{CH}_2\text{-CO}\cdot\text{COOH}$ would sometimes be formed. This on hydrolysis could yield oxalacetic acid, which is known, to say the least, to have interesting potentialities in the oxidative cycles involved in physiological oxidation.

In the *in vitro* oxidation of (I) it would be expected that $\text{RCH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CO}\cdot\text{CO}\cdot\text{COOH}$, would be formed, and this in turn converted into the triketo acid (III). This may indeed prove to be the course of the reaction, in the organism, when and if the facts can be ascertained. Under *in vitro* conditions this mechanism is to be preferred because in such homogeneous systems, where adsorption and orientation phenomena, presumably so important in heterogeneous enzyme systems, are reduced to a minimum, the enol form of the keto acid being oxidized is more readily attacked. Thus the product obtained contains two adjacent carbonyl

groups in place of the one originally present. When the adjacent keto groups reach the number of three, the molecule seems almost to fall apart with the loss of two carbon atoms. However, the details of the reaction mechanism are still unknown, even in *in vitro* conditions.

The above is enough to indicate some of the possible variants of an α -oxidation of a normal fatty acid. As a possible mechanism α -oxidation has never received the serious study that it deserves. This arose from the fact, pointed out above, that an error in organic chemistry, made many years ago, has been perpetuated in the field of fatty acid catabolism.

The β -oxidation is visualized as a similar limited multiple oxidation, that begins on the β -carbon atom, and proceeds thus:



Here again the statements made concerning α -oxidation apply, for the most part. The chief difference visualized is in the position of the successive attacks, due to the manner in which the fatty acid is adsorbed on the enzyme system, and in the nature of the intermediate triketo acids (VI) formed. In this instance the hydrolysis of (V) would give rise to acetoacetic acid. However, if (V) underwent oxidation to give $\text{RCH}_2\text{CH}_2\text{CO}\cdot\text{CO}\cdot\text{CH}_2\text{CO}\cdot\text{CH}_2\text{COOH}$ and this in turn was subjected to oxidative hydrolysis, $\text{R}\cdot\text{CH}_2\text{CH}_2\text{CO}\cdot\text{COOH}$ and acetoacetic acid would be formed. Thus in this way an initial β -oxidation could be converted into an α -oxidation cycle, with the formation of the end-product of the traditional β -oxidation. However, likewise $\text{RCH}_2\text{CO}\cdot\text{CH}_2\text{CO}\cdot\text{CH}_2\text{CO}\cdot\text{CH}_2\text{COOH}$ might be formed, although from the standpoint of organic chemistry this is also quite conjectural. Such a compound could hydrolyze to give $\text{RCH}_2\text{CO}\cdot\text{CH}_2\text{COOH}$ and acetoacetic acid, and the oxidation would continue as a β -oxidation, as conceived by some recent advocates of multiple β -oxidation.

Similarly if (V) were converted into $\text{RCH}_2\text{CH}_2\text{CH}_2\text{CO}\cdot\text{CO}\cdot\text{CO}\cdot\text{CH}_2\text{COOH}$ this on hydrolysis would give $\text{RCH}_2\text{CH}_2\text{CH}_2\text{CO}\cdot\text{COOH}$

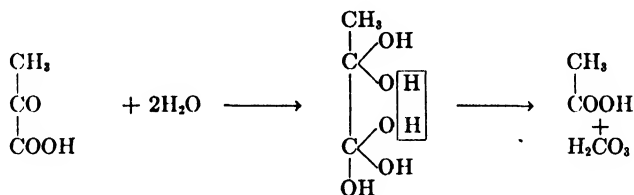
and oxalacetic acid, *i. e.*, the shift to α -oxidation would be accomplished, and no sign of β -oxidation would survive.

If (V) were hydrolyzed in the accepted traditional manner, a normal fatty acid and acetoacetic acid would be formed.

Finally it would be possible to hydrolyze (V) with the formation of a new β -keto acid and acetic acid thus: $\text{RCH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{CH}_2\text{COOH}$ and acetic acid.

If we were to consider the formation and selective oxidation of enols of intermediate keto acids the possibilities of this mode of initial attack could be considerably extended, and there is no valid reason for excluding them from the picture of possibilities.

In the above suggestive discussion of the two types of oxidative mechanisms, considerable flexibility as to details is provided, but it is assumed that the fatty acids are shortened by a hydrolytic cleavage at suitable points. Some such cleavages occur *in vitro*, without the aid of enzymes, or special catalysts, but the reaction mechanisms are so far unknown. In the organism, it may be assumed that an enzyme action is involved, but nothing is known about this. Further, for the present, it is assumed that such an enzyme or catalyst is non-specific in its action, and that it can in some degree hydrolyze various keto acids to a variable extent depending on the other circumstances. This then would provide for some of the flexibility of physiological oxidation associated with the many possibilities of α - and β -oxidation. However, such a suggestion as to the existence of enzymes for hydrolysis must be accepted with caution, because the process in the end may be a detail of the oxidation itself. For instance, pyruvic acid could be "hydrolyzed" thus:



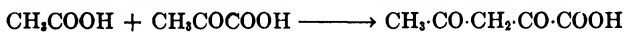
by the removal of two hydrogen atoms from the hydrate, and what appears to be hydrolysis turns out to be merely the end-result of a molecular rearrangement following a dehydrogenation.

The Recapture Synthesis Involving Acetic and Acetoacetic Acids.—

Perhaps no idea touched upon in this essay carries more important suggestions than that of the recapture of products of partial or incomplete oxidation of fatty acids. In addition to the suggestions already made on

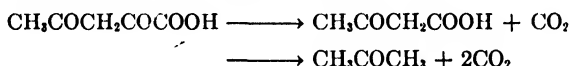
page 274,* just one possible application will be discussed in more detail.

The idea that two molecules of acetic acid are coupled end to end to give rise to either acetoacetic acid, by the loss of water, or to succinic acid by the loss of two hydrogen atoms, in the organism, has been frequently discussed. It is probably true that no one has given rigid proof for either reaction, as existing in the organism, and yet on the basis of analogous reactions and the chemical versatility of the organism, these reactions are surely possible. However, when applied to acetic acid and acetoacetic acid the general reaction has other interesting possibilities. Thus for instance acetic acid reacting with pyruvic acid would give acetopyruvic acid thus:

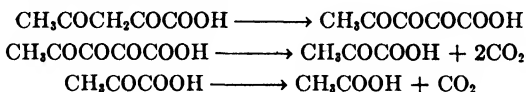


If this substance in being oxidized were converted into the triketo acid, $\text{CH}_3\text{CO}\cdot\text{CO}\cdot\text{CO}\cdot\text{COOH}$, this could readily be oxidized to $\text{CH}_3\text{COCOCH}_3$ and two molecules of carbon dioxide. The pyruvic acid so formed could again react with acetic acid, or be further converted in some other way. The chief point to be emphasized is that a resistant molecule, namely, acetic acid, has been made quite sensitive to oxidation by resynthesis.

Recently Krebs and Johnson (12) visualized this synthesis as a means of converting acetic acid into acetoacetic acid. The condensation reaction represented was the same, but the acetopyruvic acid in part was found to lose first one carbon atom, giving rise to acetoacetic acid, and this in turn by the loss of another carbon atom gave acetone, thus:



This type of reaction can also be obtained by chemical means (unpublished data). However, what appears to be much more important, it has been found in this Laboratory, that acetopyruvic acid more readily undergoes oxidation, under approximately neutral conditions, in such a way that the reaction must be interpreted as follows:



* To trace the history of this idea in relation to acetoacetic acid would require considerable space. Shaffer (18) in a long series of studies attacked the problem of such a recapture. Henze (7) continued it under simpler conditions. Others have also contributed, until it is obvious that such a synthesis occurs spontaneously. Shaffer obtained it with the "active" fragments of alkali-treated glucose; Henze obtained it spontaneously with methyl glyoxal. Henze's compound appears to be toxic, but in any case some of the possibilities have been partially canvassed.

In general, acetopyruvic acid, as is to be expected, is quite readily oxidized under circumstances in which fatty acids are resistant.

What the corresponding triketo acid, $\text{CH}_3\text{COCH}_2\text{COCH}_2\text{COCOOH}$, derived from acetoacetic acid and pyruvic acid, is like remains to be determined.

In brief these recapture syntheses provide for the conversion of two rather resistant chemical substances into highly active compounds. What has been said above is enough to indicate that the possible role of pyruvic acid in such syntheses must be thoroughly examined. A. v. Szent-Györgyi (20) states that Knoop, who gave us a fundamental picture of fat catabolism in 1904, has recently considered "that pyruvic acid represents, in fact, the great cross-road of the main-metabolic processes, not only between oxidation and fermentation, but also between fat and carbohydrate metabolism." On the authority of Knoop alone the investigation of the role of these compounds in such processes must be carried forward.

General Discussion and Résumé.—In this essay it was proposed to provide a unified hypothesis for the reciprocal integration of carbohydrate and fat catabolism. As a basis for discussion the main best known facts involving these two catabolisms were enumerated in twenty statements. On the basis of these statements it was rather evident that in order to get neutral depot fat into the zone of vital action and oxidized completely:

(a) It was necessary to provide for its transport. This was done by converting it into hydrophilic phospholipids and cholic acid-cholesterol complexes.

(b) It was necessary to bring the fatty acid into the oxidizing enzyme system of the working cells. This was done by allowing these hydrophilic complexes to enter these systems as such.

(c) It was necessary to provide two typical end-results for fatty acid catabolism, one of which provided for complete combustion, and the other for the production of acetoacetic acid, both of which are assumed to take place simultaneously, but to varying extents depending on conditions. This was done by providing an α -oxidation mechanism for the former and a β -oxidation mechanism for the latter.

(d) It was necessary to provide some means of disposing of acetic and acetoacetic acids formed, and to relate their removal to carbohydrate catabolized at the same time. This was done by a recapture synthesis, by which these relatively inert acids again become active metabolites.

In this way we come to think of four primary mechanisms of oxidation: one involving carbohydrate, two involving fatty acids and the recapture synthesis followed by oxidation. All four, it is thought, would be func-

tioning simultaneously to a variable extent in various tissues and at various times. The over-all situation in man in three typical physiological situations can be suggested by Table I. Man on a normal diet subsists largely on carbohydrate for energy requirements. In fasting, if he has good fat stores, his catabolism may shift to fat largely, for a time. In severe diabetes the shift is even more striking. The over-all integration is obtained through the fact that the same acceptor system is mediating the metabolic hydrogen, and does this on an automatic if and when basis. Carbohydrate catabolism is so adjusted that when everything is favorable the remaining mechanisms operate on an "idling" basis. However, when it fails these are brought into greater activity. In general it is to be considered that the carbohydrate catabolism is primarily dependent on phosphorylation, and when it fails in part as in more or less severe diabetes, in phlorizin poisoning, etc., the fat catabolism comes to a maximum. This suggests indirectly but clearly that fat catabolism is not a process dependent on primary phosphorylation of the molecule being catabolized, and this is supported in a general way by many considerations that have come out of the recent intensive study of the details of carbohydrate catabolism. In order to avoid bringing fat catabolism into this field, subject to control by insulin, phlorizin and the like, we have visualized the catabolism of a fatty acid as a simple dehydrogenation followed by secondary effects that lead to the formation of a ketone. One such ketone is known to be formed as acetoacetic acid, and so the idea is not entirely without support. In allowing the ketone compounds to be formed in this way we have intended merely to escape giving details that must in this case be quite hypothetical and are for the present fully unknown.

Table I
Source of Energy —————→ Mediated through Acceptor-hydrogen

	Normal Diet	Fasting	Diabetes
(1) Carbohydrate catabolism	————→	→	→
(2) α -Oxidation of fatty acids	→	————→	————→
(3) β -Oxidation of fatty acids	→	————→	————→
(4) Oxidation of recapture products	→	→	→

In Table I the length of the arrows suggests in a relative way the proportion of the hydrogen derived from the four modes of catabolism under the

conditions given, when the energy requirements are about equal for the three states. The type of material oxidized is determined by the metabolites available. In diabetes less carbohydrate is available, because of the lack of insulin. In fasting and diabetes the organism is thrown back on the oxidation of fatty acids, and the three mechanisms are indicated as working at a much higher level than would be observed under the conditions existing in a more normal individual. Because the energy of the metabolite is mediated through hydrogen acceptors, these work on a supply and demand basis, and the "preferred" position of carbohydrate catabolism is changed only by a change of the circumstances of catabolism.

It is evident that the table is schematic and that it represents an over-all picture. Thus it is obvious that different organs and tissues will show a variegated picture. This might be especially true of the liver, which could be considered, under certain conditions, to be synthesizing and mobilizing glucose, while at the same time it is burning fat for its own uses.

The chief merit of this hypothesis as sketched is that it provides for a continuity of events from the moment of the mobilization of neutral fat to the completion of the combustion, analogous to that presented in the known story of glycogen mobilization and combustion. It also provides for the reciprocal integration of these two catabolisms. All of this is accomplished by a conservative use of the best information available, and without the invention of new agents, or the contradiction of established facts. The picture as presented provides for a maximum flexibility, nor is it supposed that if one mechanism is found to exist, in fact, all others are thereby ruled out of existence. The purpose in the catalytic processes of life seems to be to overcome in a controlled way the innate "reaction resistance" of metabolites, such as glucose and fatty acids, and to control, direct and steer the chemical events involved into certain definite channels. This we have tried to provide in the hypothesis that has been sketched, but it is not assumed that this is done in but one way, and through a single channel. Just what these channels are can only be determined by enormous labor, but the general principles of the construction of such catalytic channels have been worked out in such amazing detail and clarity in the case of carbohydrate physiology, that we can scarcely expect anything less satisfactory for fats, when the story is fully told.

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In this brief essay it was not possible to trace the origin of the various ideas and facts referred to. For this purpose the older essay of Dakin, the historical treatment of Lieben and the recent book of Hilditch would be valuable, as well as the Reviews cited from the

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VITAMIN K, ITS CHEMISTRY AND PHYSIOLOGY

By

HENRIK DAM

*Copenhagen, Denmark**

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I. Introduction

Vitamin K belongs to the group of nutritional factors which are closely related to enzymes. Whereas certain other vitamins are related to carbohydrate metabolism and respiration, vitamin K has to do with the enzymes involved in blood coagulation.

The symptom of lack of vitamin K in the higher animals is bleeding tendency of a certain type. Vitamin K is therefore often referred to as an antihemorrhagic vitamin.*

Several substances have vitamin K activity. A few of these substances—vitamin K₁ from green leaves and vitamin K₂ which is formed by bacteria—occur in the food or in the intestinal content and are therefore to be considered actual vitamins, whereas others can be prepared artificially and are more or less important as substitutes for the vitamins as far as the use in therapeutics is concerned.

The description of the group of vitamin K-active substances and the role they play in the animal and plant organism are the purpose of the present report.

II. The Experimental Vitamin K-Deficiency Disease

1. General Description

The experimental vitamin K-deficiency disease was at first observed in chicks but it may be developed in all higher animals.

The disease is characterized by bleeding tendency because of low clotting power of the blood. When the clotting power of the blood is sufficiently reduced, minute lesions of vessels due to mechanical trauma are not stopped by a clot in due time but cause a continuous oozing of blood from the

* Sometimes vitamin K has been called *the* antihemorrhagic vitamin. This is obviously not quite correct because this name would just as well apply to vitamin C which also prevents hemorrhages. For the same reason one should also avoid a term like "antihemorrhagic compounds" which is sometimes used to designate vitamin K-active substances in general.

wounded part. The occurrence of the bleedings thus always presumes a trauma, but this may be so mild that its effect would scarcely be noted in the normal organism. In accordance herewith the bleedings are most frequently found on those parts of the body which are most exposed to mechanical trauma; in the chick, for instance, on legs, wings, breast, abdomen, neck and in the intestinal tract.

The clotting power of the blood may be very much reduced, but it is seldom completely lost; it is therefore often seen that the bleedings stop and are resorbed without the diet being altered and without improvement of the clotting power. In most cases, however, the animals will sooner or later die from loss of blood, if vitamin K is not supplied.

It is obvious that the bleedings may lead to a more or less pronounced anemia, and anemia is often the first sign of a suddenly developing internal hemorrhage.

Reduced gain in weight does not seem to be a feature of the K-avitaminosis.

2. *Nature of the Coagulation Anomaly*

The relation of vitamin K to the clotting process can be explained on the basis of the classical theory of blood coagulation.

This theory presumes that thromboplastin (thrombokinase) deliberated from the juice of wounded tissue cells or from disintegrated blood platelets, in the presence of Ca ions, activates a proenzyme, prothrombin, occurring in the plasma, into an enzyme, thrombin, which then converts the fibrinogen of the plasma into the insoluble fibrin.

It is easy to show that vitamin K is necessary to the maintenance of the normal amount of prothrombin in the blood plasma.

3. *The Early History of the Investigation of the Vitamin K-Deficiency Disease*

H. Dam (1929-1930) (42, 43) described the disease in chicks which were raised on an artificial diet with a very low sterol and lipid content in order to study the sterol balance. The bleeding tendency and slow clotting of the blood were noted as well as the fact that cholesterol, lemon juice, yeast or cod liver oil did not prevent the hemorrhages. W. D. McFarlane, W. R. Graham and F. Richardson (1931) (126) gave a very drastic description of the low clotting power of the blood and observed that the disease developed with a diet containing either extracted fish meal or meat meal but not with a diet in which the protein source had not been extracted in this way. W. F. Holst and E. R. Halbrook (1933) (103) also reported to have observed the disease; they found that fresh cabbage prevented the hemorrhages, but they concluded that the protective factor was vitamin C. After an interruption of his work on the subject caused by practical circumstances H. Dam (1934) (44) reported that ascorbic acid given parenterally, wheat germ oil given orally or variation of the salt mixture did not prevent the symptom

and concluded that the disease was due to the lack of a hitherto unknown factor occurring in seeds and cereals. H. Dam and F. Schoenheyder (1934) (45) studied the relation of the disease to pathological changes of the lining of the gizzard.

H. Dam (1935) (46, 47) definitely claimed that the disease was due to the lack of a new fat-soluble vitamin the properties of which were studied further. H. J. Almquist and E. L. R. Stokstad (1935) (1, 2) set forth a similar claim. They reported that the new vitamin could be formed by putrefaction and denied its relation to gizzard erosions. F. Schoenheyder (1935-1936) (151, 152) showed that blood plasma from normal chicks accelerated the clotting of plasma from K-avitaminous chicks and found that calcium, fibrinogen and thromboplastin were not reduced in quantity in the K-avitaminous chick. The indirect evidence for the lack of prothrombin provided by these observations was contradicted by the fact that it was not possible clearly to demonstrate that there was a difference in the activity of "prothrombin precipitations" from plasma of normal chicks and chicks suffering from the coagulation deficiency, and further it was found that the lipid extract of the plasma from normal chicks accelerated the coagulation of plasma from K-avitaminous chicks, so that it was likely that another factor, of lipid character, was involved instead of the prothrombin. H. Dam, F. Schoenheyder and E. Tage-Hansen (1936) (48) showed, however, that when the precipitation of the "prothrombin fraction" (with acetic acid or acetone) is carried out at low temperature, the difference in activity between the "prothrombin precipitate" from normal and K-avitaminous plasma, can easily be shown, and when the experiment is carefully made, no coagulation-promoting effect of the lipid fraction can be found. That prothrombin is low in the plasma of K-avitaminous chicks was also reported by A. J. Quick (1937) (140).

S. A. Thayer *et al.* (1937) (161), suggested that the anemia of K-avitaminous chicks was not due to hemorrhages alone but that there might be a direct effect of vitamin K on the hematopoietic system. This was very definitely contested by H. J. Almquist, E. Mecchi and A. A. Klose (1938) (11) and finds no support either in the experimental material from the reviewer's laboratory.

4. *The Diet for the Development of the Disease*

Any food composition which allows growth and maintenance of life without containing vitamin K may be used. It is not necessary that the animals should grow at the optimal rate, but it is desirable that specific symptoms of other deficiency diseases are prevented. It is worth while to keep in mind that in chicks lack of vitamin E leads to a vascular disease causing hyperemia and exudates which may be mistaken for hemorrhages (H. Dam and J. Glavind (1939) (63)). Since chicks are still most frequently being used for the study of lack of vitamin K the investigator should be familiar with the following main points of the specific dietary requirement of chicks.

Lack of vitamin E will also cause the encephalomalacia of A. M. Pappenheimer and M. Goetsch (1931) (135) (H. Dam, J. Glavind, O. Bernth and E. Hagens (1938) (61)). In order to prevent perosis ("leg weakness") the diet must contain manganese (H. S. Wilgus, L. C. Norris and G. F. Heuser

(1936) (179)) and choline (T. H. Jukes (1941) (107)). Several factors are necessary to secure optimal growth and normal development of muscles of chicks: choline, glycine, creatine and glucuronic acid (H. J. Almquist and E. Mecchi (1940) (17)).

The protein can be furnished mainly as casein, 15 to 20 per cent of the diet. Casein sometimes contains vitamin K and must then be extracted with ether or alcohol. Glycine can be given as gelatin, 8 per cent of the diet; and glucuronic acid as gum arabic, 5 per cent of the diet; the vitamin B complex can be given in the form of yeast, 10 per cent of the diet; vitamins A and D as cod liver oil, 1 to 2 per cent; vitamin E as wheat germ oil 1 to 2 per cent, or pure *dl*-alpha-tocopherol acetate about 1 to 2 mg. per day. Chicks can synthesize enough ascorbic acid to avoid scurvy. They can also synthesize nicotinic acid.

The diet can be composed on the basis of the data given above or one of the following diets can be used:

The diet described by H. Dam, J. Glavind and P. Karrer (1940) (67):

Dried and powdered ripe peas, extracted with ether	320 gm.
Dried yeast	100
Fish meal, extracted with ether	100
Saccharose	450
Cod liver oil	10
Wheat germ oil	10
Salt mixture*	10
	<hr/>
	1000 gm.

The diet of H. J. Almquist and A. A. Klose (1939) (12):

Sardine meal, ether extracted.	17.5 gm.
Dried brewer's yeast, ether extracted	7.5
Salt mixture†	1.0
Cod liver oil	1.0
Calcium carbonate	0.5
Ground polished rice	72.5
	<hr/>
	100.0 gm.

* Sodium chloride	4800
Ferric citrate	840
Copper sulfate, cryst.	60
Manganous sulfate, cryst.	300
Dijodotyrosine	0.05

6000.05

† Contains 0.5 per cent manganese as manganous sulfate.

S. Ansbacher (23) proposes a diet containing a mixture of wheat middlings and yellow corn which has been heated at 120° for one week in order to destroy vitamin K.

5. *Role of Putrefaction*

The chicks must be kept under clean conditions so that they cannot soil their food and water with feces. Even when no vitamin K is present in the diet the feces of chicks will contain vitamin K formed by putrefaction in the large intestine. The fact that the large intestine of the chick is short is believed to be the reason why only traces of the vitamin originating from putrefaction are absorbed when coprophagy and soiling of the food are prevented (cf. H. J. Ålmquist and E. L. R. Stokstad (1936) (3) and H. Dam and L. Lewis (1937) (51)).

When the chicks are kept clean, the effect of the vitamin K-free diet on the clotting power can be observed after a few days but the full development of the disease requires two to four weeks.

6. *Rats and Rabbits as Experimental Animals*

In rats and rabbits the supply of vitamin K from intestinal putrefaction apparently plays a much greater role than in chicks, ducklings and young geese. The large intestine of rats and rabbits is longer and it is known that these species eat feces directly from the anus at night. In a group of rats placed on a vitamin K-free diet many individuals become just as ill as chicks (H. Dam and J. Glavind (1939) (64), T. H. Jukes (1941) (108)), but others may resist for a very long time (64). Rabbits have been observed to get the disease only to a moderate degree (H. Dam and J. Glavind (1938) (59)).

7. *Development of the Disease by Other Means Than a Vitamin K-Free Diet*

It has been shown by W. B. Hawkins and G. H. Whipple (1935) (97) and by O. Vadsteen (1936) (170) that exclusion of bile from the intestine by bile fistula (in dogs) or choledochus ligation (in rats) leads to bleeding tendency. W. B. Hawkins and K. M. Brinkhous (1936) (98) showed that the bleeding tendency of bile fistula dogs is due to low prothrombin.

That this condition can be prevented by a diet very rich in vitamin K and therefore is probably due to lack of vitamin K, through diminished absorption of the vitamin in the absence of bile, was shown by J. D. Greaves and C. L. A. Schmidt (1937) (95).

H. Dam and J. Glavind (1938) (59) ligated the *ductus choledochus* in chicks and cured the resulting hypoprothrombinemia by intravenous injection of an emulsion of vitamin K. They further showed (1940) (69) that the effect of a given dose of vitamin K on the prothrombin level was quantitatively the same whether the disease was produced by a K-free diet or by choledochus ligation.

Further studies on the relation of vitamin K to the prothrombin deficiency in choledochus-ligated rats have been made by E. Tage-Hansen (160) and by J. E. Flynn and E. D. Warner (93).

Vitamin K deficiency due to faulty absorption of fat-soluble vitamins from the intestine can be produced in rats by giving them 20 per cent of paraffin oil in their diet (M. C. Elliot, B. Isaacs and A. C. Ivy (75a)).

A particular form of the K-avitaminosis, the hypoprothrombinemia of the newborn mammalian organism, will be discussed in relation to vitamin K deficiency in humans.

III. Determination of Vitamin K in Animal Experiments

1. General Outlines of the Assay

The determination of vitamin K can be based either on the effect of preventing visible bleedings or on the effect on the clotting power of the blood. The last-mentioned principle can be used both in prophylactic and curative tests and is supposed to be the more accurate.

A very weak vitamin K activity is best determined or detected in a prophylactic test in which the substance is given over a longer time, whereas a curative test of short duration is to be preferred for more potent substances.

A large number of methods using chicks as the test animal have been described (7, 11, 12, 21, 23, 24, 47, 58, 73, 120, 144, 152, 153, 162, 163). The use of chicks has the advantage not only that the disease is very easily developed in this species but it also simplifies the test of the blood clotting because the blood platelets of chicks and other birds do not furnish the blood with thromboplastin so that it is not necessary to take measures against spontaneous coagulation due to disintegration of blood platelets.

In the curative methods (23, 24, 58, 73, 120, 144, 153, 162, 163) which are now mostly used, the disease is developed by means of a vitamin K-free diet, whereafter the substance to be tested is given and the effect on the blood coagulation is determined after a certain time. Most methods start with day-old chicks, whereas others use chicks weighing 80–100 gm. in

order to make it possible to take larger samples of blood. It is highly advisable, of course, to test the clotting power of the blood before the test substance is given. Some methods work with only one dose of the substance, others with daily doses distributed over three days in order to obtain a more even response.

As will be fully described later the effect of one single dose of the vitamin develops gradually, reaches a maximum and then decreases. It would be an advantage if the time from the ingestion of the substance to the determination of the effect could be so adjusted that the maximum effect is obtained. This is, however, not possible to obtain for all doses, because the effect of small doses which do not render the clotting power normal reaches the maximum in a shorter time than larger doses which bring the clotting power back to normal.

Usually an interval of 18 to 24 hours after the oral ingestion of the (last) dose of the substance is used. This corresponds approximately to the maximum effect of large doses. A short interval, six hours for instance, appears to be less suitable because a bulky material like dried vegetables is not completely absorbed within that time.

The effect of the substance to be tested must be compared with the effect of a standard vitamin K preparation, if it is not intended simply to relate the activity measured to the physiological effect alone.

Among the methods for determining the clotting power the most exact are those which determine, as closely as possible, the prothrombin content but they require more work than a rough determination of the time for spontaneous clotting of blood obtained by a clip in the wing vein; this is the reason why such less exact methods are used by many investigators.

In the more elaborate methods the blood is taken either from the carotid artery—after a simple operation—through a needle without syringe (58, 153) or from the jugular vein through a very fine needle fixed to a syringe containing a measured amount of an oxalate or citrate solution which prevents the spontaneous conversion of prothrombin into thrombin due to contamination with an uncontrollable (minute) quantity of tissue extract during the outflow (E. D. Warner). When contamination with tissue juice during the outflow is completely prevented—as when the blood is taken through a needle inserted and fixed tightly into the carotid—oxalate or citrate is not necessary.

The prothrombin—that is, the clotting power as a simple function of the prothrombin—is then determined in the plasma or in the whole blood by the methods of H. P. Smith, E. D. Warner and K. M. Brinkhous (157), by A. J. Quick (140) eventually as described by H. J. Almquist and A. A. Klose (12) or by the method of F. Schoenheyder (153) as revised by H. Dam and J. Glavind (58) (see Section III, 2). The result is preferably expressed as a curve showing the variation of the prothrombin or a simple function thereof with the dose of the ingested substance calculated per gram body weight of the animal. The curve is compared with a similar curve for the standard substance.

In the simpler methods in which the spontaneous clotting power of the blood taken from a clip in the wing vein is measured (7, 11, 21, 23, 73, 120, 162, 163), the result for a given dose of the substance may be expressed as the percentage of the animals having a clotting time below (or above) a certain limit which is arbitrarily considered the limit of the normal value. The graphical plotting may be carried out by using "probits" for the ordinate and the logarithm of the dose as abscissa whereby the curve obtained with three or four doses will be more close to a straight line (120).

In view of the several changeable details it is possible to vary the assay rather widely. It is therefore not intended to present a full description of the different methods, the details of which may be obtained from the literature cited.

The most accurate method is believed to be that of E. D. Warner used in H. P. Smith's laboratory (described in a personal communication from Dr. Warner) which works with blood from the jugular vein, but it requires the most elaborate set-up. Next to this method come the methods of Dam and Glavind (58) and of Quick (144). Among the simpler methods that of S. Ansbacher (21, 23) with an interval of 18 hours from the ingestion of the substance to the final test, and an evaluation of the results as described by Lee, Solmssen, Steyermark and Foster (120) seems to be recommendable. All methods should probably be revised to work with 18 hours and one single dose.

None of the methods is very accurate. Particularly investigators working with the simpler methods have often obtained very varying results for one and the same substance and in some cases even have declared notoriously inactive substances to be as active as the pure vitamins.

It is obvious that a method working with rats would have certain theoretical advantages as compared with the chicken methods. In accordance herewith J. E. Flynn and E. D. Warner (93) have tried to use choledochus-ligated rats for vitamin K assay of substances given by the parenteral route. It would be worth while to investigate whether it is possible to develop a rat method in which the disease is produced by dietary means and complete prevention of coprophagy.

2. Remarks on the Prothrombin Determination

The method of H. P. Smith, E. D. Warner and K. M. Brinkhous (157) consists of two steps, viz.: (1) the conversion of the prothrombin into thrombin and (2) measurement of the thrombin by its action on a fibrinogen solution. The first step of the process must be preceded by a removal of the fibrinogen of the plasma. This is done by adding a small amount of purified thrombin. The excess of added thrombin is removed by letting the serum stand for a certain time before stage 2 of the process is carried out. In the second stage the thrombin originating from varying dilutions of the plasma is tested against the fibrinogen solution and the dilution which clots the fibrinogen in 15 seconds under standard conditions is measured. The same experiment is made with normal plasma whereby the result can be expressed in per cent of the normal prothrombin value; $\text{dilution}_{\text{sample}}/\text{dilution}_{\text{normal}} = \text{prothrombin per cent}$. The method can be used

for chicken blood as well as for mammalian blood because the blood is taken out in oxalate and recalcified when the prothrombin is being converted into thrombin.

The method is believed to be particularly exact for prothrombin values not too far below the normal value, because in this range the plasma is diluted so much that the influence of anticoagulants which might be present in the plasma in varying amount, is very materially reduced. The disappearance of the added thrombin is, of course, a condition for the reliability of the method, but it seems to be quite certain that this condition is fulfilled. The method has one great advantage, *viz.*, that the rate of activation of the prothrombin into thrombin is no source of error as in the one-stage methods.

The method of A. J. Quick (140) is a one-stage method. The clotting is brought about by adding a large excess of thromboplastin to the plasma.

Under this circumstance the prothrombin will be the only factor which determines the coagulation time—the prothrombin time—provided that anticoagulating substances are constant. The prothrombin time can be interpreted in terms of prothrombin by means of a standard curve obtained with mixtures of normal plasma and prothrombin-free plasma. It is not easy to distinguish between the prothrombin times corresponding to the interval 30 to 100 per cent or more of the normal prothrombin content, but if the test is carried out with plasma diluted to a known proportion the prothrombin content can be brought within the most sensitive range between 5 and 25 per cent. Usually the blood is received in oxalate, and calcium chloride is added after the thromboplastin. In this form the method is equally suitable for chicken and mammalian blood. Anticoagulating substances if present in the plasma in varying amount, are supposed to influence the result.

The method used in the reviewer's laboratory (F. Schoenheyder (1936) (153), H. Dam and J. Glavind (1938) (58, 59)) is also a one-stage method. The coagulation of the plasma is induced by means of varying dilutions of thromboplastin. Thereby the concentration K of the thromboplastin preparation which under standard conditions clots the plasma in three minutes is found. The same experiment is carried out with normal plasma whereby the concentration K_n which coagulates the normal plasma in the same time is found. The figure $R = K/K_n$ indicates the clotting anomaly and the reciprocal figure $1/R = K_n/K$, the clotting power which is approximately proportional to the prothrombin content when other factors are constant.

When the method is used for mammalian blood it is necessary to take the blood out into a small and exactly measured amount of heparin solution, whereby the spontaneous clotting due to disintegration of blood platelets is checked. A suitably small quantity of heparin does not interfere appreciably with the result so long as the prothrombin content is higher than about 1 per cent of the normal value.

The thromboplastin used in all three methods must be from a species not too different from the test animal, *i. e.*, for chicken blood the thromboplastin must have been prepared from chicken or other bird's tissue, for mammalian

blood it is necessary to use thromboplastin from mammalian (or human) tissue.

IV. Units for Vitamin K Activity

The unit for vitamin K activity can be based merely on the quantitative effect on the blood coagulation under fixed circumstances or on a comparison with the corresponding effect of a certain amount of a standard substance. The following units have been proposed:*

	A	B	C	D
	Equivalent in 2-methyl-1,4- naphtho- quinone, microgram	Equivalent in vitamin K ₁ , microgram	Equivalent in 2-methyl-1,4- naphthohydro- quinone- diacetate, microgram	Equivalent in Dam-Glavind units
H. J. Almquist and A. A. Klose (12), "Reference standard:" 1 cc. of hexane extract of dried alfalfa equals 1 gm. of dried alfalfa	4.2 (13)	16 (13)	...	100 calculated from A
S. Ansbacher (21): the mini- mum amount necessary to render the clotting time of the K-deficient chick weigh- ing 70-100 gm. normal (less than 6 minutes), 6 hours after the administration	0.5 (24)	20 by direct assay in 6- hour test (21), 12.5 calculated from A
H. Dam and J. Glavind (58): 2 mg. of a dried and pow- dered spinach stored in the form of tablets. The effect on blood coagulation is equal to 1 Schoenheyder unit	0.04 (67)	0.083 (67)	0.071 (67)
P. F. Dann (73): a concen- trate from alfalfa	0.4 (73)	1 (73)	...	25 ? (73), 10 where calcu- lated from A

(Table continued on page 296)

* The equivalents of the different units in terms of known substances are based on the values from the literature which are considered most reliable, but it should be kept in mind that most investigators have indicated different values at different times. References are presented in parentheses.

	A	B	C	D
	Equivalent in 2-methyl-1,4- naphtho- quinone, microgram	Equivalent in vitamin K ₁ , microgram	Equivalent in 2-methyl-1,4- naphthohydro- quinone- diacetate, microgram	Equivalent in Dam-Glavind units
F. Schoenheyder (153): that quantity calculated per gm. body weight of the chick which given in 3 days renders the clotting power normal	1 (58)
S. A. Thayer (163): that quantity which when given to K-avitaminous 24-day-old chicks on each of 3 successive days produces a clotting time of 10 minutes or less in 50 per cent of 10 or more chicks	0.95 (165)	About 2 (cf. 166)	2 (29)	24 to 28 calculated from A, B and C

The following chemical compounds have been proposed as standard substances for vitamin K: 2-methyl-1,4-naphthoquinone (165); 2-methyl 1,4-naphthohydroquinone-diacetate (78, 67). The latter compound is more stable toward light and air oxidation than the quinone and still more stable than vitamins K₁ and K₂ or the diacetates of their hydroquinones. The Ansbacher and Almquist-Klose units have been much used by clinical workers in the United States, but the Dam-Glavind or Schoenheyder unit has been more used in all-round research on vitamin K and is defined by a more exact method. There is no obvious reason for the use of the aforementioned units instead of those used by the original investigators in this field.*

V. Occurrence of Vitamin K in Nature

In the plant the principal source of vitamin K is the green leaf and all other chlorophyll-containing plant organs (several hundred Dam-Glavind units per gm. dry weight). Most fruits are poor sources—an exception is

* But it seems obvious to define in the future these units by means of one of the easily accessible pure compounds, say, the diacetate of 2-methyl-1,4-naphthohydroquinone. The relative strength of this compound and vitamins K₁ and K₂ should be measured as exactly as possible. A logical alternative to the original units would be to calculate the activity of a given substance in terms of microgram of one of the natural K-vitamins.

tomatoes. Cereals, beans and ripe peas contain little of the vitamin, and in carrots, potatoes and mangolds the content is practically zero (57).

Certain bacteria are very rich in vitamin K (10, 18, 72, 133) whereas yeast is practically vitamin K-free (47).

In the animal organism vitamin K does not occur so abundantly as in the plant. Only small quantities are deposited in the different organs of the hen, even if the food is rich in natural sources of the vitamin (60). The liver is no particular place of deposit, neither does much vitamin K pass into the eggs. The mammalian organ which has hitherto been found to contain most vitamin K is hog liver—about 50 Dam-Glavind units per gm. dry weight. Cow's milk and human milk are very poor sources, less than one-third unit per cc. ((136) and unpublished data from the reviewer's laboratory). The feces are rich in vitamin K. A man who in five days had lived on a practically vitamin K-free diet still excreted vitamin K in the feces—2000 Dam-Glavind units per gm. fecal lipid (59). It is not surprising that the urine does not contain vitamin K even when the diet is rich in natural vitamin K (59).

VI. Chemistry of Vitamin K and Related Compounds

1. *Isolation, Characterization, Structure and Synthesis of the Natural K-Vitamins*

Immediately after the discovery of vitamin K, its chemical properties were studied mainly by H. Dam and co-workers and by H. J. Almquist and his group. These two groups of workers showed that vitamin K can be highly concentrated by extraction with lipoid solvents, separation of less active material by freezing and purification of the more active fraction by selective adsorption and molecular distillation (47, 49, 50, 51, 53, 60, 2, 4, 5, 6, 8, 9, 14).

H. J. Almquist showed that the vitamin is sensitive to light (4). Very strong concentrates from alfalfa were made after the method described by H. Dam, J. Glavind, L. Lewis and E. Tage-Hansen (1938) (60). Having improved this method further, H. Dam, A. Geiger, J. Glavind, P. Karrer, W. Karrer, E. Rothschild and H. Salomon (1939) (62) reported isolation of the vitamin in the form of a light yellow oil, the characteristics of which were presented. The elementary composition was found to be C 82.2 per cent, H 10.7 per cent and O 7.1 per cent. The absorption maxima in the ultraviolet region were found to be 243, 248, 261, 270 and 328 millimicron. The extinction coefficient at 248 millimicron $E_{1\%}^{1\text{cm.}} = 280$. The substance gave a color reaction with sodium ethylate consisting in the de-

velopment of a violet-blue color which slowly turns to red and brown. The biological activity was estimated at 20 million Dam-Glavind units per gm., a figure which was later corrected to 12 million (67). Sjögren and Sundberg (156a) found 12-14 million D-G-units per gm.

H. J. Almquist and A. A. Klose (14) reported isolation of vitamin K as a choleic acid melting at 186-189° from which the vitamin could be liberated as a viscid oil. No criterion for the purity of this product was set forth.

From the work of the California and the Copenhagen groups it was known that the activity of vitamin K from alfalfa was destroyed by saponification agents and by bromine, but not by acetylating agents or ketone reagents.

P. Karrer and co-workers took up the investigation of the constitution (110, 111, 112, 113) but before their final results were published two other groups of workers succeeded in clearing up the configuration.

At St. Louis University (E. A. Doisy's Laboratory), D. W. MacCorquodale, *et al.* (123), and R. W. McKee, *et al.* (127), isolated vitamin K both from alfalfa and from putrefied fish meal. In accordance with Dam, Karrer and co-workers (62) they reported that vitamin K from alfalfa is an oil but they found that vitamin K from putrefied fish meal is crystalline (m. p. 54° (128, 31)). They introduced the terms vitamin K₁ for vitamin K from alfalfa (which is supposed to be the form of vitamin K manufactured by the green leaf in general) and vitamin K₂ for the vitamin K formed by putrefaction.* Detailed descriptions of methods for preparing vitamin K₁ from alfalfa were published by Karrer, *et al.* (112), and by S. B. Binkley, *et al.* (28). Detailed description of the method for purification of K₂ is found by R. W. McKee, *et al.* (128).

The quinoid structure of vitamin K₁ was recognized by R. W. McKee, *et al.* (127), and by P. Karrer and A. Geiger (110). The quinoid structure of vitamin K₂ was reported by R. W. McKee, *et al.* (127).

This finding led several workers to test various quinones for vitamin K activity. H. J. Almquist and A. A. Klose (16) found that phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone), which occurs in the tubercle bacillus, had a slight vitamin K activity, and S. Ansbacher and E. Fernholz (22) found a very high activity for 2-methyl-1,4-naphthoquinone.

* P. Karrer and A. Geiger (110) in agreement with H. Dam proposed the name *phyloquinone* for vitamin K from green leaves. As long as a nomenclature comprising all vitamin K-active naphthalene derivatives (not only naphthoquinones) has not been adopted, it seems reasonable to use the terms K₁ and K₂ along with the name *phyloquinone* for vitamin K₁. A corresponding name for vitamin K₂ referring to its origin and occurrence could easily be introduced, in case it is desired to replace the terms K₁ and K₂ by names.

D. W. MacCorquodale *et al.* (124) showed that ozonolysis of the diacetate of the hydroquinone corresponding to vitamin K₁ yielded a ketone, 2,6,10-trimethyl-pentadecanone 14. Oxidation of vitamin K₁ with chromic acid yielded among other products two acids: phthalic acid and supposedly 2-ethyl-1,4-naphthoquinone-3-acetic acid. These observations might be in agreement with the structure of 2-ethyl-3-phytyl-1,4-naphthoquinone for vitamin K₁. Later, however, S. B. Binkley *et al.* (30), and D. W. MacCorquodale, *et al.* (125), found that the acid was not the ethyl but the corresponding methyl compound. The condensation of phytyl bromide with the monosodium salt of 2-methyl-1,4-naphthohydroquinone yielded 2-methyl-3-phytyl-1,4-naphthohydroquinone which could be converted into the corresponding quinone by shaking with air. This compound turned out to be identical with natural vitamin K₁.

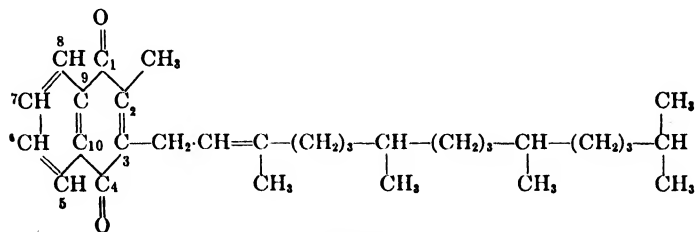
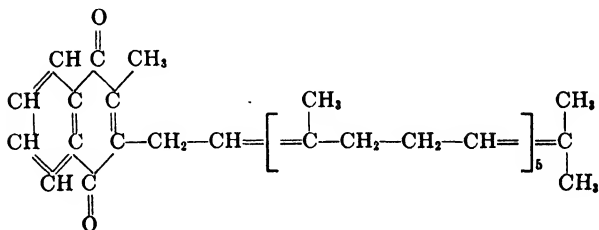
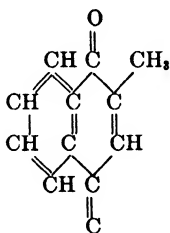
L. F. Fieser and co-workers (83, 84, 85, 87, 88) prepared pure vitamin K₁ from a concentrate made from alfalfa by B. Riegel, *et al.* (146).

The essential feature of the purification method of Fieser (87, 88) consisted in the conversion of the vitamin present in the concentrate into the corresponding hydroquinone by hydrosulfite whereby treatment with alkali can be carried out without destruction of the vitamin. The vitamin-hydroquinone sodium salt can be taken down into alcoholic KOH from petroleum and after dilution with water it can be extracted with ether.

Fieser and co-workers further assayed a number of substituted naphthoquinones for vitamin K activity and compared the ultraviolet spectra and sodium ethylate reactions of such compounds and natural vitamin K₁. They synthesized 2-methyl-3-phytyl-1,4-naphthoquinone by heating phytol and a large excess of 2-methyl-1,4-naphthohydroquinone in the presence of water-free oxalic acid. The resulting 2-methyl-3-phytyl-1,4-naphthohydroquinone was purified and oxidized by silver oxide to the quinone. The corresponding ethyl compound was found to be inactive.

R. W. McKee, *et al.* (127), found the elementary composition of vitamin K₂ to be 84.43–84.7 per cent carbon; 9.87–9.73 per cent hydrogen. By catalytic hydrogenation vitamin K₂ took up 18 atoms of hydrogen as compared with 8 atoms for vitamin K₁. The absorption spectrum and the color reaction with sodium ethylate were practically as for vitamin K₁. Vitamin K₂ is also a 3-substituted 2-methyl-1,4-naphthoquinone (S. B. Binkley, *et al.* (31)), because ozonization of vitamin K₂ in glacial acetic acid and decomposition of the ozonide with ether and zinc yield 1,4-diacetoxy-2-methyl-naphthalene-3-acetaldehyde which can also be obtained from vitamin K₁. The degradation with KMnO₄ resulted in the formation of phthalic acid. The side chain in K₂ is much more readily oxidized than the phytyl group in K₁. Ozonization of vitamin K₂ gives as a water-soluble

product levulinaldehyde in the proportion of up to 5 mols per mol vitamin K_2 . On the basis of this observation the following formula was proposed for vitamin K_2 :

Vitamin K_1 Vitamin K_2 

2-Methyl-1,4-naphthoquinone

Before these experiments were made another formula had been set forth for K_2 , *viz.*, 2,3-difarnesyl-1,4-naphthoquinone (L. F. Fieser, *et al.* (85)), but this formula was not based on direct experimental evidence and it was disputed by P. Karrer and A. Epprecht (114) who suggested a formula for K_2 not very different from that which was later found by S. B. Binkley, *et al.*

The activity of vitamin K_2 has been estimated at 8-9 million Dam-Glavind units per gm. (67).

2. Other Vitamin K-Active Compounds

The Activity of Simple Derivatives of Vitamins K₁ and K₂.*—If vitamins K₁ and K₂ are converted into the diacetates of the corresponding hydroquinones, the activity is somewhat reduced (29, 67). This apparently also holds true for the diphosphates or disulfates (89).

Hydrogenation of the double bond in the phytyl group of K₁ results in a lowering of the activity (67, 92).

Other side chains may be introduced in the 3-position but it appears that a branched structure with a double bond in the same position as in the phytyl group in K₁ and containing 20–30 carbon atoms is particularly favorable to the activity ((92), see also (67)).

The 2,3-oxide of vitamin K₁ is highly active (92).

Replacement of the methyl group by hydrogen results in a considerable loss of activity (164, 67).

Replacement of the long side chain by hydrogen (2-methyl-1,4-naphthoquinone) results in an increase of activity as calculated per gm., whereas the activity calculated on a molar basis is not much different for the three compounds: vitamin K₁, vitamin K₂ and 2-methyl-1,4-naphthoquinone (67, 73, 156a).

The Activity of Simple Derivatives of 2-Methyl-1,4-Naphthoquinone.—Conversion into the diacetate of the corresponding hydroquinone reduces the activity moderately (164, 67). The corresponding disuccinate (67) and disulfate (89) are also supposed to be moderately weaker than the quinone itself. The diphosphate has been said to be particularly active (120).

Introduction of hydroxyl adjacent to the methyl group reduces the activity very much (phthiocol) (16, 164).

The same holds true for introduction of alkyl substituents in the benzenoid ring (92, 67).

Replacement of the methyl group in the 2-position by hydrogen (164, 67), ethyl (92, 156) or propyl (92) results in an enormous fall in activity. According to M. B. Moore (129a) SO₃Na may be introduced in the 3-position in 2-methyl-1,4-naphthohydroquinone without inactivation.

Partial or complete hydrogenation of the benzenoid ring results more or less in a drop of activity according to the site of the hydrogenation (92). According to L. F. Fieser, M. Tishler and W. L. Sampson (92) the 5,6,7,8-tetrahydro compound is very little active as compared with the 5,8,9,10-tetrahydro or 5,8-dihydro compound.

* Due to the inexactness of the methods used by most investigators and the varying results laid down in the literature it is not attempted to list absolute values for the different compounds.

The Activity of Other Quinones, Naphthols and Naphthyl Amines.—1,4-Naphthoquinone gives much more active derivatives than any other quinone tested even if certain other quinones are slightly active (124, 67, 117a).

1-Naphthols (92), 1-amino-naphthalenes (92), 4-amino-1-naphthols (74, 67) and tetralones (92) having a methyl group in the 2- or 3-position are also highly active.

It is possible that such compounds as well as many of the above-mentioned more or less active substances are converted by the organism into 2-methyl-1,4-naphthoquinone or a derivative of this substance which is responsible for the vitamin K effect. This possibility has been discussed by L. F. Fieser, *et al.* (92). It might be that the many different vitamin K-active compounds are in fact to be considered provitamins for vitamin K, but it is not easy to provide direct experimental evidence for this view. It must also be kept in mind that F. Bernheim and M. Bernheim (27) have found that a compound like 2-methyl-4-amino-1-naphthol has a catalytic activity of the kind which has been considered for vitamin K in an *in vitro* system without tissue cells (see Section VIII, 3).

The slight but demonstrable effect of a more remote compound like anthraquinone (67) could just as well be supposed to be due to the compound itself as to the conversion into a 2-methyl-1,4-naphthoquinone.

A short description of a few vitamin K-active substances other than vitamins K₁ and K₂ which have been used for experimental and clinical purposes is given below.

Vitamin K₁ hydroquinone diacetate prepared by reductive acetylation of the vitamin (29, 110), white crystals, m. p. 59°, soluble in fat solvents, approximately half as active as the vitamin (29). Treatment with methyl-magnesium-iodide and subsequent shaking with air convert the substance into vitamin K₁.

Vitamin K₂ hydroquinone diacetate (29) white crystals, m. p. 57–58°, soluble in fat solvents, approximately half as active as vitamin K₂.

Vitamin K₁-2,3-oxide, obtainable by treatment of the vitamin with hydrogen peroxide (91), almost colorless oil, soluble in fat solvents, approximately as active as vitamin K₁ (92). Sodium hydrosulfite converts it into the corresponding hydroquinone.

2-Methyl-1,4-naphthoquinone, obtainable by CrO₃ oxidation of 2-methyl-naphthalene (129, 90), yellow crystals, m. p. 106°, sensitive to light, soluble in ether, alcohol, acetone, glacial acetic acid, slightly soluble in water, 25,000 Dam-Glavind units per mg. (67), about twice as active as vitamin K₁. (Sjögren and Sundberg (156a) found 30,000–35,000 D-G-units; Hepding and Moll (101a) 35,000.)

2-Methyl-1,4-naphthohydroquinone diacetate, obtainable by reductive acetylation of the quinone (129), white crystals, m. p. 115.5°, soluble in fat solvents, stable in light, 14,000 Dam-Glavind units per mg. (67), approximately as active as vitamin K₁. (The activity found by Sjögren and Sundberg (156a) is 20,000 D-g-units. Hepding and Moll (101a) found 14,000.)

2-Methyl-1,4-naphthohydroquinone disuccinate, white powder, soluble in water at slightly alkaline reaction, 15,000 Dam-Glavind units per mg. (67).

2-Methyl-1,4-naphthohydroquinone diphosphate tetra sodium salt, $6H_2O$, obtainable from the hydroquinone by esterification with $POCl_3$ and pyridine, etc. (89, 94, 120), white crystals, water-soluble, supposed to be half as active per mg. as the quinone, and 50 per cent more active than the quinone when calculated on a molar basis (120).

2-Methyl-1,4-naphthohydroquinone disulfate disodium salt, $2H_2O$, obtainable from the hydroquinone by esterification with chlorosulfonic acid and pyridine, etc. (89, 90), white crystals, water-soluble, rather potent (89).

2-Methyl-1,4-naphthoquinone-2,3-oxide, obtainable by treatment of the quinone with hydrogen peroxide (90), white crystals, m. p. $95.5-96.5^\circ$, soluble in fat solvents. Sodium hydrosulfite converts it into the corresponding hydroquinone. Supposed to be considerably less active than the quinone (92).

A solution prepared by dissolving *2-methyl-1,4-naphthohydroquinone* in warm sodium bisulfite, supposed to contain 2-methyl-3- SO_2Na , 1,4-naphthohydroquinone, approximately as active as 2-methyl-1,4-naphthoquinone (129a).

Phthiocol, 2-methyl-3-hydroxy-1,4-naphthoquinone, obtainable by isomerization of 2-methyl-1,4-naphthoquinone-2,3-oxide with sulfuric acid (90), orange crystals, m. p. $172-173^\circ$, soluble in alcohol, ether, acetone, slightly soluble in water. The vitamin K activity is low (16, 164).

2-Methyl-naphthoquinone-monooxime, light yellow crystals, m. p. 165° , soluble in ether, acetone, alcohol, 5000 Dam-Glavind units per mg. (67).

2-Methyl-4-amino-1-naphthol hydrochloride, red powder, water-soluble (67, 74, 76), 1000 Dam-Glavind units per mg. (67).*

2-Methyl-1-amino-4-naphthol hydrochloride, has similar properties as the just mentioned compound and is nearly as active (76).

Regarding the other, simpler vitamin K-active substances—methyl naphthols, methylnaphthylamines, tetralones, etc.—the reader is referred to the handbooks of chemistry.

All the free quinones mentioned above, 2-methyl-1,4-naphthoquinone, for instance, have as far as they are even slightly water-soluble a very marked burning taste and irritate the mucosa of mouth and intestinal tract; if they are given in large quantities they may cause vomiting, albuminuria or porphyrinuria. In the natural vitamins the long side chain gives these compounds a very marked lipid character. This slows down the burning taste as well as all reactions in the aqueous phase of the cell. The esters of the hydroquinones do not possess the burning taste either, as long as they are not slightly decomposed. This should be kept in mind when compounds are chosen for clinical use.

* Some authors have used the name K_2 for this compound. Such a name appears to be rather arbitrary because the compound does not belong to the group of naturally occurring K-vitamins but to the big group of artificial vitamin K substitutes. The nomenclature would be unnecessarily complicated, if all these substances should be named by the letter K and an index.

3. Redox Potentials of Some 1,4-Naphthoquinones

It is likely that the action of vitamin K in the organism has to do with the redox properties of the quinoide groups (see Section VIII, 3). Therefore the redox potentials of vitamin K₁ and a few other compounds are listed below:

	E ₀ at pH 0, millivolt	Temp., ° C.	Reference	Approximate vitamin K activity, Dam-Glavind units per mg.
Vitamin K ₁	363	20	(147)	12,000
	328	22	(122)
2-Methyl-1,4-naphthoquinone	408	25	(82)	25,000
	422	25	(121)
	458	22	(122)
Phthiocol	300	30	(25)	50-100
	256	22	(122)
1,4-Naphthoquinone	484	..	(82)	50
2,6-Dimethyl-1,4-naphthoquinone	405	..	(82)	"0"

It is apparent that the redox potential of a certain 1,4-naphthoquinone derivative is not the only factor which determines the vitamin K activity (compare particularly 2-methyl-1,4-naphthoquinone and 2,6-dimethyl-1,4-naphthoquinone).

VII. Determination of Vitamin K by Physical or Chemical Methods

1. Principles Available

Several principles are available as a basis for physical or chemical determination of vitamin K in plant or animal organs:

- (A) The ultraviolet absorption of the vitamins or certain easily obtainable derivatives of them.
- (B) Polarography.
- (C) Color reactions.
- (D) Catalytic hydrogenation of the quinones and subsequent titration of the hydroquinones formed in this way with a suitable hydrogen acceptor.

(A) The ultraviolet absorption can only be used for strong concentrates of the natural vitamins or for the artificial compounds of known chemical character and extinction coefficient.

The absolute value for the extinction at 248 millimicron for vitamins K₁ and K₂ has been indicated as follows:

	E1 % 1 cm.	Reference
Vitamin K ₁	280	(62)
	385	(127)
	540	(29)
	448	(166)
	387*	(86)
Vitamin K ₂	305	(128)

* Calculated from $\log \epsilon = 4.24$.

(B) E. B. Hershberg, J. K. Wolfe and L. F. Fieser (102) showed that vitamin K₁ can be determined polarographically in aqueous isopropyl alcohol containing KCl. They found a sharply defined wave at minus 0.58 volt. As little as 50 micrograms of the vitamin dissolved in 2.5 cc. isopropyl alcohol could be determined accurately.

(C) The color reaction with sodium ethylate or methylate can be used for strong concentrates but it has the disadvantage that the color is unstable. H. J. Almquist and A. A. Klose (1939) (15) used the red-brown stage of the color and eliminated the carotenoids by extraction with a hydrocarbon solvent. The theoretical explanation of this reaction has been given by P. Karrer (111) and by L. F. Fieser, *et al.* (85). The reaction is characteristic for such 1,4-naphthoquinone derivatives which, like vitamins K₁ and K₂, have a side chain in the 3-position containing a double bond between the β - and γ -carbons. This double bond activates the —CH-group attached to carbon 3 in the quinoid ring in such a manner that it can react with alkali and give rise to mesomeric forms by shifting of the double bond 2-3 in the quinoid ring to the 1-2 position. The later red or brown stages are due to a splitting off of the side chain resulting in the formation of phthiocol.

The utilization of the red-brown color for the determination of vitamin K is dependent upon how quantitatively and reproducibly this last stage of the reaction is proceeding. If this condition is fulfilled, the reaction can be used for the estimation of the total number of molecules of vitamins K₁ and K₂ in a mixture of these two vitamins.

It has been proposed to use certain other color reactions for naphthoquinones in the determination of vitamin K (130) but apparently without any efforts to ensure specificity or practical usefulness of the reactions proposed.

F. Irreverre and M. X. Sullivan (106a) have described a colorimetric test based upon the reaction between vitamin K (or other 2,3-substituted

1,4-naphthoquinones) and sodium diethyl dithiocarbamate in alkaline alcoholic solution, whereby a transient blue color is developed. The intensity of the color is about 5 times that of the sodium ethylate reaction. Other naphthoquinone derivatives give a pink, red, brown or green color.

(D) N. R. Trenner and F. A. Bacher (169) and G. V. Scudi and R. P. Buhs (154a) have described a method and the particular apparatus suitable for catalytic hydrogenation of the quinones and subsequent titration of the hydroquinones with 2,6-dichlorophenol-indophenol. The substance to be tested is dissolved in 95 per cent *n*-butanol and a little phenosafranin is added. Raney's nickel serves as catalyst. The end-point of the hydrogenation is shown by the disappearance of the pink color of the phenosafranin, the redox potential of which is much lower than that of the K-vitamins. The 2,6-dichloro-indophenol is also dissolved in 95 per cent butanol. Vitamins A and E do not interfere with this method, but tocoquinone which is a common oxidation product of vitamin E, will interfere, if present. The lower practical limit of the amount of vitamin K₁ titratable in this way is believed to be 100–20 micrograms according to the size of the apparatus.

2. Extraction Methods

The extraction of vitamin K from dried plant material is effected by means of fat solvents under the necessary precaution against excessive heating, exposure to light and air during the evaporation of the solvent. H. Dam (54) reported that more vitamin K could be found in the extract than could be determined directly by feeding the dried vegetable, apparently because the vitamin is more effectively extracted *in vitro* than in the intestinal tract.

L. F. Fieser (87, 88) has, as previously mentioned, introduced the step of purifying the extract in the presence of hydrosulfite whereby the vitamin is reduced to the hydroquinone stage. Under these circumstances saponification can be carried out without destruction of the vitamin. This procedure has been used by Scudi and Buhs (154a) in preparing purified extracts for the chemical determination.

VIII. Mode of Action of Vitamin K in the Animal Organism

1. The Time Factor

As mentioned in Section II, 2, vitamin K is necessary to the maintenance of the normal amount of prothrombin in the blood.

When vitamin K is given intravenously it is possible to study the effect

at different intervals from the moment of the introduction into the blood stream. It is thereby revealed that the action does not set in instantaneously but requires a certain time for its development (H. Dam, J. Glavind, L. Lewis and E. Tage-Hansen (1938) (60). If the prothrombin content of the blood of a K-avitaminous chick is about 1 per cent of the normal value at the starting point, it takes about five hours to raise it to between 50 and 100 per cent of the normal, assuming that a sufficient amount of the vitamin (natural vitamin K₁) is injected. The prothrombin increases further to about 100 per cent during the following hours and is in most cases in the neighborhood of 100 per cent 24 hours after the injection. Thereafter it falls again unless very great quantities have been given, in which case the prothrombin may stay normal for a few days before a marked fall sets in. If smaller quantities are injected than necessary to bring the prothrombin up to the normal value, then the fall sets in more rapidly (60, 69). Corresponding results have been found in experiments with oral ingestion of the vitamin (S. Ansbacher (20, 21)).

2. *The Organ Involved*

When vitamin K is added to the blood from a K-avitaminous animal *in vitro* no improvement of the prothrombin content is observed, even if the vitamin remains in contact with the blood for five or six hours at body temperature. This observation suggests that the action of the vitamin takes place in tissue cells (60).

The most logical way of examining the importance of a certain organ to the action of vitamin K would be to extirpate the organ from a K-avitaminous animal, introduce vitamin K intravenously and follow the action. Such an operation on a K-avitaminous animal involves, of course, a considerable risk of bleeding and is therefore difficult to carry out. Organs which are not essential to life may, however, be removed before the animal is given the K-free diet. In this way it is easy to show that the spleen is not essential to the action of vitamin K (60).

When the liver is removed from a normal or slightly K-avitaminous gander and the blood coagulation is examined by methods also affected by variations in anticoagulating substances in the blood, an improvement of the coagulating power is found as the result of the removal of the liver (60). When similar experiments are carried out with methods more specific for the prothrombin a decrease of the prothrombin is recorded in some hours. The improvement found in the first-mentioned experiments must be due to

diminished output of anticoagulating substances (unpublished experiments from the reviewer's laboratory).

Andrus, Lord and Moore (1939) (19) have ectomized the liver in normal dogs and studied the level of prothrombin in the blood with and without ingestion of vitamin K and bile salts. They found that the prothrombin decreased in both cases.

Several other observations show that the liver is concerned with the formation of prothrombin. Thus E. D. Warner (1938) (175) reported a decrease in prothrombin after removal of two-thirds of the liver in rats. Intoxication involving severe damage to the liver also led to a fall in prothrombin. This is found, for instance, after ingestion of chloroform (H. P. Smith, E. D. Warner and K. M. Brinkhous (1937) (157)). Vitamin K does not prevent or cure the hypoprothrombinemia met with in these intoxications (K. M. Brinkhous and E. D. Warner (1940) (35)). Further, it is known that vitamin K does not cure the hypoprothrombinemia in patients with Laennec's cirrhosis or Banti's disease, where the liver parenchyma is severely damaged (150).

The relation of vitamin K to the hypoprothrombinemia caused by the toxic substance in spoiled sweet clover (Schofield (154), Roderick (149), Stahmann, Huebner and Link (158) and Butt, Allen and Bollman (40)) has not yet been sufficiently cleared up. Certain earlier experiments described by C. K. Drinker and K. R. Drinker (1916) (75) suggest that the bone marrow also is involved in prothrombin formation. This problem has been subjected to a renewed study by Barnes (25a) who found that extensive destruction of the bone marrow by x-ray irradiation did not affect the prothrombin formation.

3. *Nature of the Action*

As to the way in which vitamin K affects the formation of prothrombin two alternatives must be considered. The first is whether vitamin K is a constituent of prothrombin. So far as is known prothrombin is a protein; it accompanies the globulins in many precipitation reactions and does not dialyze. Therefore, and because of the time factor in the development of the effect, vitamin K cannot be identical with prothrombin. But one could imagine that vitamin K might enter the prothrombin molecule as a prosthetic group. This is, however, not very likely. If vitamin K was present in the prothrombin molecule, one would expect that prothrombin itself would act as vitamin K, so that the peroral ingestion of prothrombin would cure the hypoprothrombinemia of K-avitaminous animals. Experiments in which prothrombin was precipitated from large amounts of normal hen's

plasma—at pH 5.3—did not show any certain vitamin K activity when given to small chicks living on a vitamin K-free diet (60). The most likely explanation of the action of vitamin K is therefore that the vitamin enables certain cells to produce prothrombin (60).

The mechanism of this action is unknown. It is likely that the redox properties of the quinones play a role in an enzyme system which has to do with the formation of prothrombin. McCawley and Gurchot (122), F. Bernheim and M. Bernheim (27) and J. Percy Baumberger (26) have made suggestions in this direction: —S—S— groups are believed to be an essential constituent of the prothrombin molecule, and as shown by Bernheim and Bernheim, naphthoquinone derivatives (2-methyl-1-hydroxy-4-amino-naphthalene) may catalyze the formation of such groups from —SH groups in cysteine *in vitro*.

IX. Vitamin K Deficiency in Humans

1. The Simple Alimentary K-Avitaminosis

This condition is rare. Most food articles are not entirely free of vitamin K and, what is more important, under normal conditions there is an ample supply of vitamin K from putrefaction processes in the intestine.

R. Kark and E. L. Lozner (1939) (109) were the first to report cases of alimentary K-avitaminosis (moderate hypoprothrombinemia) in humans living on a very restricted diet which also gave rise to other deficiency symptoms. E. D. Warner, T. D. Spies and C. D. Owen (176) examined the prothrombin in poorly nourished patients in Alabama, but they were unable to find signs of K-avitaminosis associated with pellagra, the common deficiency disease in this district, unless a severe diarrhea occurred which accounted for a diminished absorption of the vitamin from the intestine.

2. The Cholemic Bleeding Tendency

The cholemic bleeding tendency which affords a great danger in operations on patients with obstructive jaundice was the first hemorrhagic condition in man which was recognized to be due to lack of vitamin K.

Prothrombin deficiency in connection with cholemic bleeding tendency due to obstructive jaundice has been observed by A. J. Quick, *et al.* (139), and Quick (140) suggested the possibility that this hypoprothrombinemia was caused by diminished absorption of vitamin K in the absence of bile from the intestine. That this is the true explanation of the bleeding tend-

ency in patients with obstructive jaundice was shown independently by E. D. Warner, K. M. Brinkhous and H. P. Smith (175a), by H. R. Butt, A. M. Snell and A. E. Osterberg (37) and H. Dam and J. Glavind (1938) (55). Similar observations have been made with patients with bile fistula (see 34, 183).

The constituents of the bile which are of importance to the absorption of fat-soluble vitamin K as well as other fat-soluble vitamins are the bile acids. It has been shown by Ravdin, Johnston, Riegel and Wright (145) and by Breusch and Johnston (32) that the bile acid content of the bile is low during the first one or two weeks after the patient has been operated on and the bile returned to the intestine. This explains why it is often observed that the bleeding tendency develops after the obstruction has been relieved. It is also of importance in this respect that the food intake and the content of the intestine are minimal in the days after the operation whereby the formation of vitamin K by bacteria is reduced.

Various authors have fully established the fact that vitamin K treatment completely eliminates the risk of bleeding in connection with the operation of patients with obstructive jaundice. Compare the reviews of K. M. Brinkhous (36), H. R. Butt and A. M. Snell (39), F. Koller (117), B. Riegel (148) and the thesis of E. Tage-Hansen (160).

The treatment of the bleeding tendency consists of giving the patient vitamin K one or two days before and a certain time (two weeks, for instance) after the operation in such a way that its transmission to the blood stream is secured. If natural fat-soluble vitamin K is given orally, bile acid, for instance, desoxycholic acid, must be given simultaneously with the vitamin. Such vitamin K substitutes which are more or less soluble in water may be given orally without bile acid, or parenterally. The dose may be 1 Dam-Glavind unit per gm. body weight per day (159, 160). The esters of the hydroquinones should be preferred to 2-methyl-1,4-naphthoquinone (116, 117) (see Section VI, 2). Overdosage does not afford any danger of intravascular clotting because vitamin K cannot raise the prothrombin above the normal value, but extreme overdosage of certain of the artificial vitamin K-active compounds should be avoided (see Section VI, 2).

An important point is the determination of the prothrombin along with the treatment. The vitamin K therapy is known to be so safe that in most cases the control of the prothrombin is not strictly necessary, and vitamin K therapy should be used also in cases where the prothrombin is normal before the operation because of the fall in prothrombin and resulting bleeding tendency after the operation. This might seem to render the control of the prothrombin unnecessary. Nevertheless a complication of the ob-

structive jaundice with certain changes of the liver may block the action of the vitamin (38, 150) and in such cases the operation will be dangerous in spite of the vitamin K treatment. This is the reason why the effect of the vitamin should be controlled. At the beginning the more elaborate but also more exact laboratory methods of Smith, Warner and Brinkhous (157), A. J. Quick (140) or Dam and Glavind (55, 59) were used for the determination of the prothrombin. Later a series of bedside tests and simple micro methods became available (81, 104, 115, 136, 142, 157, 182 and others).

The principle of most of these tests is to bring a small amount of blood taken from the ear or heel into contact with a similar quantity of thromboplastin, in a micro test tube or on a glass slide, and to determine the clotting time. It is possible to make the starting point of the coagulation sharper by taking the small amount of blood out in heparin (136) or in citrate or oxalate and then add CaCl_2 after the thromboplastin (118).

The interpretation of the coagulation time in terms of prothrombin can be made by means of curves showing the coagulation time, under the conditions of the test, for mixtures of normal blood and prothrombin-free blood.

3. Hemorrhagic Diathesis Associated with Intestinal Diseases

After the finding of the K-avitaminosis in connection with obstructive jaundice and complete bile fistula, it was to be expected that this condition would be found also in connection with other diseases where the absorption of fat and fat-soluble vitamins is reduced. The hemorrhagic diathesis met with in certain cases of *sprue* (coeliac disease) was therefore suspected to be a K-avitaminosis. The relation to one of the vitamins was considered by Fanconi (see 79, 80) before the discovery of vitamin K. Engel (1938) (77) showed the presence of hypoprothrombinemia in sprue in infants, and Hans Hult (1939) (106) definitely demonstrated the response to vitamin K. F. Koller found vitamin K deficiency in sprue in adults (116a). Clark, Dixon, Butt and Snell (1939) (41) have also found the K-avitaminosis in connection with sprue and, further, in cases of *colitis ulcerosa*. The K-avitaminosis in these diseases is caused by insufficient absorption due to the abnormal condition of the intestinal epithelium and to the rapid passage through the intestine. Such cases should be treated parenterally.*

4. Hypoprothrombinemia of the Newborn

The most frequent form of the K-avitaminosis in man is that of the newborn baby.

* In this connection it is interesting to note that Baldwin, Wiswell and Jankiewicz (24a) found that vitamin K greatly reduces the mortality among chicks suffering from coccidiosis.

Cases of low clotting power of the blood of infants were occasionally observed many years ago (G. H. Whipple (1912 and 1913) (177, 178); very low prothrombin was found in a case of *melenia neonatorum*. In recent years K. M. Brinkhous, H. P. Smith and E. D. Warner (33) showed that the low clotting power which may be found in newborn is due to low prothrombin, and they showed that in an infant with actual bleeding the prothrombin was particularly low. The first report of the fact that this condition is common in newborn in the first week and that it could be prevented with vitamin K came from W. W. Waddell, *et al.* (1939) (171, 172). Independent observations of the same kind were made by L. M. Hellman, *et al.* (99, 155), H. Dam, E. Tage-Hansen and P. Plum (65, 66), K. K. Nygaard (131) and A. J. Quick and Grossman (141). This field was further studied by these and many other authors especially after micro methods for the examination of the blood coagulation had been introduced (26a, 94a, 96, 100, 101, 104, 105, 115, 118, 119, 132, 134, 137, 138, 149a, b, c, d, 173, 174, 180 and others).

The result of all these investigations is that in most newborn babies the prothrombin is lower than in normal adults already at birth. During the first three days after birth the prothrombin decreases further, reaches a minimum and then rises again. Approximately normal values are found between one and two months of age or later, but the most sudden rise takes place within a few days after the minimum is passed, and the baby is in most cases out of danger of bleeding at the end of the first week after birth.

There is a seasonal variation in the prothrombin values in the blood of the newborn: the low values are more frequent during the later winter months than during the summer (149c, 137, 174).

The reason why the prothrombin of the baby is low at birth (as well as in fetal life) is not fully understood. According to the investigations of E. Tage-Hansen (160) and O. Thordarson (167) the mother's prothrombin at the time of delivery is higher than the normal value for adults whereas at the same time the baby's prothrombin is low. If vitamin K is given to the mother a suitable time before delivery the baby's prothrombin will be much higher at birth but not always normal (between 20 and 100 per cent of the value for normal adults)(118). Ingestion of vitamin K or a water soluble vitamin K substitute to the baby apparently causes somewhat higher prothrombin values than are obtained by treatment of the mothers. This might indicate that the placenta in some way or other offers resistance to the transmission of vitamin K from the mother to the fetus (137), but it remains to be determined whether the content of vitamin K is actually lower in the blood of the fetus than in the mother's blood. If this should be found not to be the case, then other factors must be taken into consideration. It is worth while to keep in mind that the prothrombin is particularly low in prematurely born babies (99) and in cases where the mother has a

pronounced albuminuria (137) whereas the normal, physiological icterus of the newborn does not aggravate the hypoprothrombinemia (137).

It has been suggested by L. Tocantins (168) that the hypoprothrombinemia of the newborn is due to low functional capacity of the liver. If this is the true explanation then this incapacity must be different from the low functional capacity of the liver met with in liver cirrhosis and chloroform intoxication, because in these latter cases the low prothrombin production cannot be increased by increased supply of vitamin K.

It is not surprising that the prothrombin falls further during the first days after birth, because there is practically no supply of vitamin K from bacteria in the intestine, and the milk, which is taken only in small quantities, is a very poor source of vitamin K (about or less than 0.3 Dam-Glavind unit per cc. (137)).

According to Quick and Grossman (143) the reason why the prothrombin rises again after three to four days must be sought in the development of the bacterial flora. As will be described in Section XI the production of vitamin K depends largely upon *B. coli*, whereas lactic acid bacteria, for instance, produce much less. The production of vitamin K by the bacteria occurs in the cell and not in the substrate. The transmission of the vitamin from the bacteria to the intestinal wall must therefore apparently be due to the death and partial disintegration of the bacteria.

L. Salomonsen and K. K. Nygaard (149b) and Salomonsen (149d) observed that when cow's milk was given to the baby very early, the hypoprothrombinemia could be checked more or less. This is not in contrast to the conception that the bacterial flora is the decisive factor, because the protein of cow's milk is not so easily digested as mother's milk, a circumstance which will favor the development of the putrefaction bacteria.

Sells, Walker and Owen (154b) hold a view quite different from that of Quick and Grossman (143) and of Tocantins (168). They consider the very low vitamin K content of the milk sufficient for the baby and estimate the daily requirement of vitamin K at as little as 1 microgram per day. In contrast to these authors F. Koller (117) is of the opinion that the need of vitamin K is particularly high for babies.

It is obvious that a thorough investigation of these conflicting explanations is necessary.

Most babies pass through the short period of low prothrombin without developing hemorrhages, but if they are subjected to surgical operation or rough handling, in the period that the prothrombin is less than 20 to 25 per cent of the normal value for adults, they will be in danger of bleeding.

The percentage of babies with actual bleeding in the first week is about

one per cent. This figure is considerably lowered by vitamin K treatment of the mothers or by treatment of the babies immediately after birth. Cases of bleeding from the umbilicus, the skin or the intestinal tract may be successfully prevented or cured in this way. The number of cases with intracranial hemorrhages may also be cut down.

According to the recent statistics of Dr. L. M. Hellman at Johns Hopkins Hospital the total death rate among newborn in this hospital has been cut from 4.5 to 1.8 per cent by treatment of the mothers 24 to 2 hours before delivery with 1 dose of 2 mg. 2-methyl-1,4-naphthoquinone (personal communication from Dr. Hellman).

Cases of lack of vitamin K have also been observed in infants aged one or two months suffering from *icterus gravis* (65). A full explanation of the origin of this form of K-avitaminosis has not yet been given.

5. *The Possible Relation of Vitamin K to Other Hemorrhagic Diseases*

It is not intended to discuss here all the hemorrhagic conditions, which have no relation to vitamin K. It should be mentioned, however, that no such relation exists in *hemophilia* (52, 59, 150) or in *thrombocytopenia* (59, 150). That vitamin K does not prevent *scurvy*, is obvious. Vitamin K has no relation either to the hemorrhages in *ablatio placenta* (70) or in *gastric ulcer* (unpublished investigations by H. Lebel and the reviewer). Sheely (154c) has recently reported that *hemoptysis* in patients with pulmonary tuberculosis may in some cases be accompanied by lowered prothrombin, which may be influenced by Vitamin K. He ascribes the reduction of prothrombin to the toxemia resulting from the tuberculous infection. Gyntelberg and Dam (96a), however, did not find any significant decrease of prothrombin in a series of cases of hemoptysis. Lack of Vitamin K cannot, therefore, be the principal cause of hemoptysis in pulmonary tuberculosis. E. D. Warner (paper read at the Symposium on Vitamins, Chicago, Sept., 1941) has occasionally found lowered prothrombin in cases of *pernicious anemia* but this form of hypoprothrombinemia was influenced by aqueous liver extract.

6. *Vitamin K Treatment as a Test for Liver Function*

The fact that certain pathological changes of the liver are accompanied by hypoprothrombinemia which cannot be corrected by vitamin K treatment can be utilized for the clinical examination of liver function. Severe damage to the liver parenchyma (in Laennec's cirrhosis and Banti's disease,

for instance) is, at least in its later stages, accompanied by an irreversible hypoprothrombinemia (38, 150, 117, 120a). Acute hepatitis may be accompanied by a moderate hypoprothrombinemia (55, 160) which according to some authors (160, 117) may be corrected by vitamin K treatment, whereas obstructive jaundice which is not complicated by damage to the liver parenchyma leads to a hypoprothrombinemia which always responds to vitamin K. Low prothrombin which is not materially raised within 24 hours after the intravenous introduction of, say, 10 mg. of one of the water-soluble esters of 2-methyl-1,4-naphthohydroquinone, may therefore be taken for a safe criterion of severe liver injury. On the other hand, normal prothrombin does not necessarily mean that the liver is normal because other tests may indicate hypofunction of the liver before the prothrombin is affected (38, 150, 120b).

It is interesting to note that in most clinical cases of impaired prothrombin formation due to liver damage the formation of fibrinogen is not materially affected. On the other hand conditions have been observed where fibrinogen production is blocked without influence on the prothrombin (in cases of *ablatio placentae* (70)). It is not absolutely certain that impaired fibrinogen formation is a sign of liver injury.

X. Role of Vitamin K in the Green Plant

The green leaf, as well as other chlorophyll-containing plant organs, is a principal source of vitamin K, whereas plant organs which normally do not contain chlorophyll are poor sources or are completely free from the vitamin.

As far as is known, the form of vitamin K in the green leaf is vitamin K₁, 2-methyl-3-phytyl-1,4-naphthoquinone. In leaves, needles of the coniferae or the thallus of seaweed, vitamin K₁ occurs in the amount of some hundred Dam-Glavind units per gm. dry matter. Dried spinach leaves, for instance contain 500 units or 40 micrograms of vitamin K₁ per gm. (H. Dam and J. Glavind (1938) (57)). The vitamin is found in the chloroplasts but not in the cytoplasm of the plant cell (H. Dam, J. Glavind and N. Nielsen (1940) (71)).

When the leaf withers in the fall vitamin K does not disappear rapidly: green chestnut leaves contain approximately 800 units per gm., and approximately the same amount is present in yellow and brown leaves during the withering process (57).

This is not surprising because vitamin K₁ and chlorophyll are very dif-

ferent in structure except for the phytyl group, and vitamin K is therefore not attacked by the enzymes which are active in the conversion of the chlorophyll.

The yellow spotted leaves of *Codiaeum variegatum* contain approximately the same amount of vitamin K in the green and yellow parts (57), and there is also about the same amount of vitamin K in leaves from normal *sambucus canadense* as there is in leaves of sambucus trees having the inheritable anomaly of low chlorophyll content (unpublished data from reviewer's laboratory (1941)). This shows that vitamin K may be formed in normal amount without simultaneous occurrence of chlorophyll synthesis to the normal extent.

In white spotted coleus leaves there is a big difference in vitamin K between the white and green areas (100 units against 500 units) (unpublished data from reviewer's laboratory), indicating that when no chloroplast pigments are formed the vitamin K synthesis is considerably diminished.

The low vitamin K content of carrots and yellow corn indicates, however, that vitamin K synthesis is not necessarily connected with the formation of carotene, xanthophyll or cryptoxanthine.

Balance experiments with peas grown in the light and in darkness, respectively, show that there is practically no synthesis of vitamin K in the pea plant grown in the dark where chlorophyll is not formed, whereas an abundant amount of vitamin K is synthesized in the pea plant grown in the light (57).

Corresponding experiments carried out with *picea canadense*, a conifera which can form chlorophyll in the dark, showed that this plant can also synthesize vitamin K without light (71).

All this tends to show that the formation of vitamin K in the higher plant is connected with certain stages of the formation of chloroplasts but is not necessarily dependent on a complete formation of normal chloroplasts.

Whether the formation of phytyl is the part of the process which limits the formation of both chlorophyll and vitamin K in the pea plant grown in the dark, may only be a guess and is not very likely. Part of the phytyl radicle is also contained in tocopherol, and this substance is found in large amount in apple pips (H. Dam, J. Glavind, I. Prange and J. Ottesen (1941) (68)), which are fairly well protected against light and contain no chlorophyll. Protochlorophyll which is formed to some extent in the dark also contains phytyl. It has been found that iron is necessary to vitamin K formation in the plant (unpublished data from reviewer's laboratory (1941)).

Vitamin K occurs somewhat differently in the plant than vitamin E. Both substances are found in green leaves (in the chloroplasts) but vitamin E is found in abundant quantity in many plant organs which have little or no vitamin K (the lipid from hips and apple pips, wheat germ oil).

The significance of vitamin K to the function of the chloroplasts is just as unknown as the significance of the carotenoids. It may be, however, that the redox properties of the substance play a role in the plant cell similar to what has been suggested for vitamin K in the animal organism. It is likely that vitamin K has something to do with the photosynthesis.

It has been found that vitamin K decreases in amount in the crushed content of the plant cell (71). This observation might suggest the existence of enzymes or compounds in the plant cell, probably in the cytoplasm, which are capable of converting vitamin K in some way or other.

XI. Role of Vitamin K in Saprophytes and Heterotrophic Unicellular Organisms

Among the saprophytes mushrooms have been examined for vitamin K (57) and found to be very poor in this substance, if they contain it at all. This is in agreement with the assumption that vitamin K is connected with photosynthesis.

The unicellular heterotrophic organisms provide an interesting field for vitamin K research.

Whereas yeast is practically free from vitamin K (40 per cent of dry yeast may be present in the diet without noticeable effect on the blood coagulation) certain bacteria are rich sources. As previously mentioned, the fact that vitamin K may be formed by putrefaction was first reported by H. J. Almquist and E. L. R. Stokstad (1). H. J. Almquist, C. F. Pentler and E. Mecchi (1938) (10) assayed several bacteria and found vitamin K production by means of a number of putrefaction organisms and no vitamin K formation by *Pseudomonas aeruginosa*. Later data for vitamin K in various bacteria have been published by H. J. Almquist (1941) (18). Formation of vitamin K by the flora of the rumen in the cow was reported by McElroy and H. Goss (125a), H. Dam, J. Glavind, P. Plum and Mejlbo (unpublished data from reviewer's laboratory) found 1000–3000 Dam-Glavind units per gm. dry matter in *B. coli* grown on a vitamin K-free pepton-broth, and practically no vitamin K in *B. acidophilus*. H. Dam, J. Glavind, S. Orla-Jensen and A. Orla-Jensen (72) and S. Orla-Jensen, A. Orla-Jensen, H. Dam and J. Glavind (133) investigated the capacity of vitamin K synthesis for *B. coli* and certain lactic acid bacteria isolated

from feces of babies and adults, and grown on a vitamin K-free substrate. The results were as follows:

	Dam-Glavind units per gm. dry matter
<i>B. coli</i>	750-1600
<i>B. bifidum</i>	Up to 300
<i>Streptococcus fecium</i>	Up to 300
<i>Microbact. lact.</i>	300

Thus *B. coli* was found to be the most active vitamin K producer. The vitamin was found in the bacteria themselves and not in the substrate.

The formation of vitamin K by *B. coli* is not dependent upon the presence of a particular "provitamin," since the formation of vitamin K proceeds to the same extent in an entirely synthetic medium consisting of glucose, asparagin, ammonium citrate and salt mixture as in a substrate of pepsin-digested casein and yeast autolysate. Suggestions about the existence of a provitamin (23) are lacking direct experimental evidence.

What purpose the vitamin K synthesis in the bacteria may serve is unknown. An important step toward the explanation of the role of vitamin K in the bacteria was the discovery of D. W. Woolley and J. McCarter (1940) (181) that 2-methyl-1,4-naphthoquinone and phthiocol are growth factors for the Johne's bacillus.

In the experiments of Dam, Glavind and Nielsen (71) with yeast cells no stimulative effect upon growth or respiration could be found.

It seems likely that a microbiological test for vitamin K may be worked out.

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THE ADRENAL CORTICAL HORMONES

By

J. J. PFIFFNER

Detroit, Mich.

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The partial synthesis of desoxycorticosterone by Steiger and Reichstein in 1937 (1) has served as a powerful stimulus to the more recent investigations in adrenal physiology both in the laboratory and in the clinic. The results of many of these studies have made it increasingly clear that certain of the hormones of the adrenal cortex differ significantly in their effects on the animal organism. The accumulated facts offer evidence in a corollary manner for the existence in adrenal extracts of at least one hormone in addition to the five now known to have cortin activity.

In this article the writer has attempted, first, to summarize the progress which has been made in our understanding of the chemistry of the cortex hormones and, second, to correlate these advances with those physiological observations which serve as the basis for the rather generally accepted view that a hormone of a high order of activity yet remains to be isolated. The problem of the intermediary metabolism of the adrenal steroids is considered briefly. No effort has been made to present either the chemistry or the physiology in an inclusive fashion. In view of the limitations of space and the defined objective of this report many important contributions to both aspects of the problem are not cited. The historical background has been entirely neglected. Mention should be made, however, of the early physiological studies of Stewart and Rogoff (2), Banting and Cairns (3), Hartman (4) and Swingle (5) and their associates. Their painstaking work did much to establish on a sound basis the survival of the adrenalectomized cat and dog as an accepted criterion of biological potency. Their results have been analyzed by Britton (6) and more recently by Grollman (7).

In examining the evidence for the existence of another highly active hormone in cortex extracts it is necessary to discuss comparative physiological activities. This is a difficult undertaking. Much of the information is of a fragmentary nature due in part to the relatively small quantities of the pure hormones other than desoxycorticosterone which have been available for physiological study. Methods of assay are numerous and varied in principle. For the most part they have not been widely standardized. Some workers have found it difficult to reproduce the assay results of others. It has been only since Robert Loeb's discovery (8) of the close association of sodium metabolism with adrenal function that the necessity of dietary control in assay work has been generally appreciated. Certain of the test procedures used in the past in fractionation work and assumed to give parallel results are now known to measure different types of physiological activities, a fact which was not recognized until synthetic desoxycorticosterone became available for study. These are some of the factors which must be weighed in evaluating statements in the literature on the relative biological activity of cortex preparations.

I. Nomenclature

The name *cortin* was proposed in 1928 by Hartman and his collaborators (9) for the hormone of the adrenal cortex necessary for the maintenance of life in the adrenalectomized animal. This term was adopted by many workers and used synonymously with *adrenal cortex extract*. More recently it has been employed in a more restricted sense by Hartman and Spoor (10) and by Wells and Kendall (11) in referring to fractions of the total extract after certain other physiological factors had been separated. Continued use of the term synonymously with various glandular concentrates may give

rise to confusion. The value of the term in the opinion of the writer rests in its connotation of a specific physiological response. In this paper *cortin activity* indicates the ability to keep adrenalectomized animals alive.* As compounds both active and inactive were isolated from adrenal extracts by different workers they were arbitrarily assigned trivial laboratory designations, such as *A, B, C, et cetera*. These laboratory terms assigned by different workers, the use of which was essential to clarity in early isolation studies, have been amply correlated by Reichstein (12, 13), Strain (14), Wintersteiner and P. E. Smith (15), Mason, Hoehn and Kendall (16), Callow (17) and others. In view of the rapid elucidation of the structures of both the active and inactive compounds by Reichstein and by Kendall and their associates the use of these letter designations is no longer necessary and for the sake of simplicity of discussion will not be referred to in this article.

The first compound isolated from the adrenal gland which had significant cortin activity was named *corticosterone* by Reichstein (18, 19). Other cortin-active constituents were later named according to their structural relation to corticosterone, namely, dehydrocorticosterone, 17-hydroxycorticosterone, 17-hydroxydehydrocorticosterone and desoxycorticosterone. After separating the above compounds as well as many cortin-inactive steroids from adrenal cortex extracts a cortin-active sirup remains which will be referred to as the *amorphous fraction*.

II. Hormone Concentrates

It is not necessary for the purpose of this article to consider preparative procedures in any detail. All extracts which have been used for chemical study have been made from whole beef adrenal glands. It is now generally accepted that neutral water-miscible organic solvents such as alcohol or acetone extract the maximum yield of hormones. The numerous methods of extract preparation differ only in the means of separating the hormone mixture from ballast material and adrenalin. In all the procedures an aqueous concentrate is produced by distilling off the organic solvent under reduced pressure at a low temperature.

* Recently Selye (*Science*, **94**, 94, (1941)) has suggested the term corticoid to signify "adrenal cortical hormone-like." Although a brief term such as this is desirable for simplicity of expression it may be misleading unless used to signify only maintenance of life in the adrenalectomized animal. Several steroids, for example, have no cortical activity but are "corticoids" in the sense that they cause salt and water retention in the normal dog.

In the method of Swingle and Piffner (20) the hormones are extracted from the aqueous concentrate with benzene and the acetone-soluble portion of the benzene extractives is partitioned between diluted alcohol and petroleum ether. Traces of adrenalin are removed by filtering the alcohol-soluble fraction through permutit. The alcohol-soluble fraction is suspended in water and a small amount of relatively inert water-insoluble material removed by filtration. In further purification work (21, 22, 23) the total alcohol-soluble fraction was used as such. Grollman and Firor (24) extract the aqueous concentrate with benzene, wash adrenalin from the benzene solution with dilute acid and after suspending the benzene-soluble material in water, filter off and discard the water-insoluble fraction. More recently Grollman (25) clarifies the aqueous concentrate by chilling and filtering and then extracts the hormone mixture with ethyl acetate. Some inert material is separated by further fractionation with methyl alcohol-ethyl acetate mixtures. Cartland and Kuizenga (26) wash the aqueous concentrate first with petroleum ether to separate lipid material and then wash the hormone mixture into ethylene dichloride. This fraction is then partitioned between diluted alcohol and petroleum ether. The water-soluble portion of the diluted alcohol fraction constitutes the extract. Kendall (27) filters off the suspended material in the first aqueous concentrate and then extracts the hormone mixture with chloroform. The chloroform-soluble fraction is suspended in water and the inert water-insoluble fraction removed by filtration. The yield of tissue extractives by these various methods is about 25 to 75 gm. from one ton of glands. An aqueous solution of this type of material represents the *adrenal cortex extract* used in most investigations of adrenal cortex physiology. Further purification depends upon the fact (21) that the cortin activity can be washed back and forth between ether or benzene and water, the distribution coefficient being 1 to 3 or 4 in favor of water. Weak acid or alkali may be used in the aqueous phase to trap impurities. This principle of purification was employed with various modifications by Piffner, Wintersteiner and Vars (22), Reichstein (23), and Mason, Myers and Kendall (28). Further fractionation with neutral organic solvents yielded a variety of crystalline products and a cortin-active amorphous fraction (20, 26).

Reichstein (23), Wintersteiner and Piffner (29) and, more recently, Kuizenga and Cartland (30) applied Girard's reagent (31) under mild conditions and separated the cortin activity in the ketone fraction. A method of fractional acid hydrolysis of the ketonic complex at room temperature was introduced by Reichstein (32). This procedure proved an exceptionally valuable one for the isolation of crystalline compounds since the several ketonic fractions were then quite readily crystallizable in part. However, the cortin activity was scattered among the ketonic fractions. Most of it remained in the non-crystallizable portion giving rise to an *amorphous fraction* comparable in a general way to that mentioned above.

Reichstein and von Euw (33, 34) applied a half-ester separation with succinic anhydride and also used chromatographic adsorption methods extensively on a variety of fractions in the isolation of crystalline compounds. In such experiments, however, no observations were made on the distribution of cortin activity.

This brief summary is given to indicate the gentle nature of the steps employed in preparing hormone concentrates which to date have yielded five cortin-active compounds as well as a large number of related steroids. Isolation with such mild methods is in favor of the view that all of these compounds are natural constituents of the gland.

III. Isolation and Characterization of Steroid Constituents

1. *Compounds with Cortin Activity*

Early in 1937 the isolation of a pure compound having cortin activity was announced by de Fremery, Laqueur, Reichstein, Spanhoff and Uyldert (19). They suggested the name corticosterone since other evidence which will be discussed below had already indicated its steroid nature. About two months later Kendall, Mason, Hoehn and McKenzie (35) reported an independent isolation of the same substance with observations on its biological activity. Both Reichstein (32) and Mason, Myers and Kendall (28) had described the isolation of this compound in impure form in 1936 with no observations, however, on physiological potency. The molecular formula of $C_{21}H_{30}O_4$ was assigned to the pure compound by Reichstein (18) and by Mason, Hoehn, McKenzie and Kendall (36). The former characterized the compound through numerous ester derivatives and the latter workers described a hexahydro product, $C_{21}H_{36}O_4$. Both groups of workers recognized the compound as an α - β unsaturated ketone with an α -ketol grouping. Considerable difficulty was encountered in characterizing the fourth oxygen atom but it was eventually shown to function as an unreactive secondary hydroxyl group (18, 35, 37).

Dehydrocorticosterone was isolated from the cortex by Mason, Myers and Kendall (28) in 1936 and by Reichstein and von Euw (33) in 1938. The former workers first assigned the formula $C_{26}H_{36}O_6$, but in the following year they (36) gave the formula as $C_{21}H_{28}O_4$ and recorded some observations on the physiological activity of the compound. A dihydro derivative was prepared and an α -ketol and an α - β unsaturated ketone group shown to be present. The fourth oxygen was assigned a hindered carbonyl function. In the meantime Reichstein (18) had converted corticosterone, $C_{21}H_{30}O_4$, to dehydrocorticosterone, $C_{21}H_{28}O_4$, through the acetate by chromic acid oxidation, thus proving conclusively the close relationship of these two compounds, but as noted above he did not isolate dehydrocorticosterone from the gland until some time later.

In the course of preparing pure corticosterone from the crude crystallisate Reichstein (18) in 1937 isolated 17-hydroxycorticosterone and the isolation was later achieved also by Mason, Hoehn and Kendall (16). Its formula, $C_{21}H_{30}O_6$, was established by Reichstein (18, 38) who also showed the substance to be an α - β unsaturated ketone with an α -ketol side chain of two carbon atoms, in common with corticosterone and dehydrocorticosterone. The compound was readily oxidized to a ketone, $C_{19}H_{24}O_3$ (adrenosterone),

and was converted by mild oxidation of its acetate to the acetate of the previously known 17-hydroxydehydrocorticosterone, $C_{21}H_{28}O_5$.

The last mentioned compound was isolated by Wintersteiner and Pfiffner (29) in 1936 and also by Reichstein (32) but they failed to recognize its physiological activity. Mason, Myers and Kendall (28) isolated the substance at about the same time in larger quantity and clearly demonstrated the cortin activity of the compound. The molecular formula, $C_{21}H_{28}O_5$, was established and its α - β ketonic character recognized (28, 29, 32). Reichstein (38) and Mason, Myers and Kendall (28) demonstrated the occurrence of a dihydroxy acetone grouping with two of the carbon atoms comprising a side chain. The fifth oxygen was characterized as a hindered carbonyl function (16, 38).

In the course of a careful systematic examination of a relatively inactive side fraction, Reichstein (33) in 1938 isolated a fifth cortin active compound, desoxycorticosterone, $C_{21}H_{30}O_4$, which he and Steiger (1) had synthesized the previous year. In the same fractionation experiment (33) 17-hydroxydesoxycorticosterone, $C_{21}H_{30}O_4$, was also isolated. No data are available on the cortin activity of the later compound but mention of its isolation is made because it can be expected to have some slight cortin action. In common with the other five active compounds it was shown (29) to be an α - β unsaturated ketone with an α -ketol two carbon side chain since it yielded 4-androsten-3,17-dione on chromic acid oxidation. More recently it has been identified with the synthetic product (39).

The amounts of these compounds obtained from the gland are very small. On the basis of 1000 pounds of glands the approximate yields in milligrams are as follows: corticosterone, 333 (27), 340 (33); dehydrocorticosterone, 333 (27), 6 (33), 23 (30); 17-hydroxycorticosterone, 37 (18), 34 (30); 17-hydroxydehydrocorticosterone, 500 (27), 200 (33), 250 (29), 85 (30); desoxycorticosterone, 12.5 (33); 17-hydroxydesoxycorticosterone, 6 (33). Considering the great similarity in the properties of these compounds and the complexity of the material from which they are obtained it is unlikely that the amounts isolated bear any real relation to the concentration in the gland. On the other hand, it should be emphasized that neither desoxycorticosterone nor 17-hydroxydesoxycorticosterone has been shown to be present in the main active fraction.

2. Compounds with Sex Hormone Activity

A relationship of the adrenal cortex to the gonads was long suspected on clinical grounds but there was no supporting chemical evidence available until 1936 when Reichstein (40) isolated an α - β unsaturated triketone of

the composition $C_{19}H_{24}O_3$. It had about 20 per cent of the activity of androsterone as measured on the growth of the capon's comb. He named the compound adrenosterone. More recently he has isolated (33) 11-hydroxyisoandrosterone, which is about one-thirtieth as active as androsterone on the capon's comb (41). Another constituent of the gland of interest in this connection is 17-hydroxyprogesterone which was found to have androgenic activity in the castrate rat (42). Progesterone (43) and oestrone (44) have been isolated from cortin-inactive side fractions. None of these compounds, however, has been demonstrated to occur in the main cortin-active fraction.

3. Physiologically Inactive Related Compounds

No useful purpose would be served in discussing the isolation and characterization of the many compounds since shown to be closely related to the hormones. They are incorporated in Table I. The saturated pentol, $C_{21}H_{36}O_5$, was isolated by Wintersteiner and Pfiffner in 1935 (45). It was the first pure compound of this series to be obtained from the gland. Reichstein (23) secured the compound a short time later in relatively large amount and his work on this inactive substance laid the foundation for the elucidation of the structure of the entire group. The substance $C_{21}H_{36}O_5$ yielded a tetra- or penta-acetate, a mono-acetone derivative and was precipitable with digitonin (23).

Properties of the Amorphous Fraction.—The cortin-active sirup which is obtained in the course of separating the crystalline hormones has chemical properties closely resembling those of the isolated crystalline hormones, *e. g.*, solubility, specific ultraviolet absorption, reaction with ketone reagents, reducing properties, fluorescence reaction, sensitivity to alkali, *et cetera*. It has no distinguishing chemical characteristic. In fact all the evidence indicates that it is a complex mixture of compounds closely related to the corticosterone group. On the other hand, it should be emphasized that neither the presence nor the absence of the known hormones in such a highly active sirup has as yet been satisfactorily demonstrated. A report that the cortin activity in the amorphous fraction disappeared on continued fractionation with organic solvents has not been elaborated upon (27). The yield of the amorphous fraction from one thousand pounds of beef glands is of the order of 1 to 3 gm. depending on the type of purification employed (12, 22, 27, 29, 30).

IV. Structure

The structures of the known cortex hormones have been almost entirely cleared up as a result of the researches of Reichstein and of Kendall and their colleagues. The first suggestive bit of evidence for the steroid nature of the new highly oxygenated compounds from the cortex was the observed

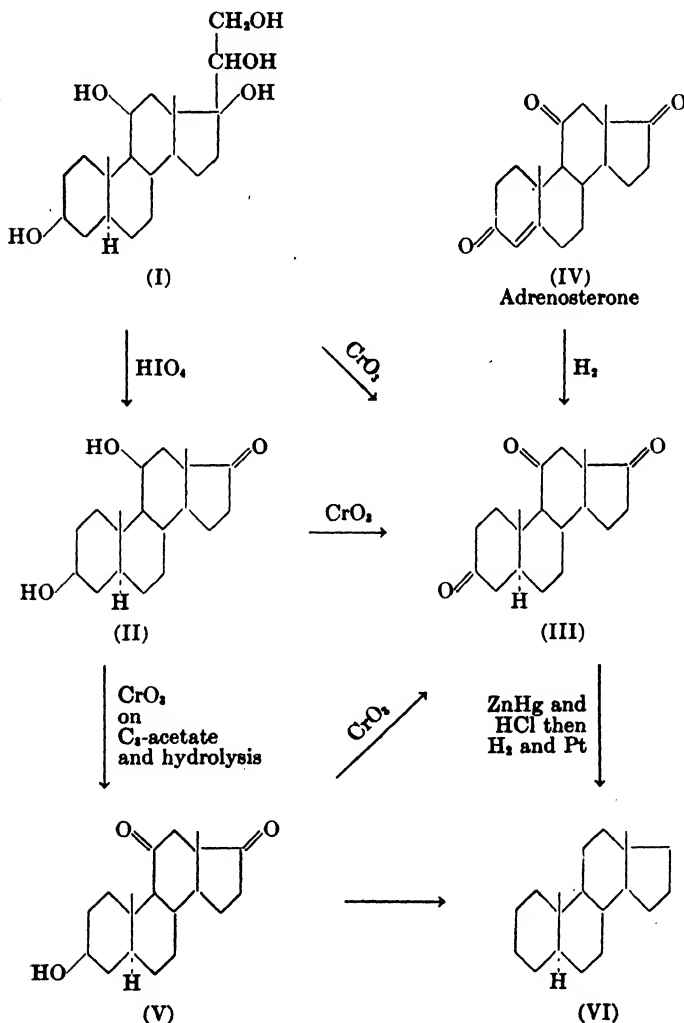
TABLE I
STEROIDS ISOLATED FROM THE ADRENAL GLAND^a

(No. 11 is corticosterone; No. 14, dehydrocorticosterone; No. 5, 17-hydroxycorticosterone; No. 6, 17-hydroxydehydrocorticosterone; No. 19, desoxycorticosterone; No. 12, 17-hydroxydesoxycorticosterone)

No.	Formula	Structure ^b	M. p., ° C.	[α] _D (Absolute alcohol)	References (Isolation and structure)
1	C ₂₁ H ₃₀ O ₆	Allopregnan-3(β), 11, 17(β), 20, 21-pentol	221-222	+16	16, 23, 28, 38, 45, 41, 37
2	C ₂₁ H ₃₀ O ₆	Allopregnan-3(β), 11, 17(β), 21-tetrol-20-one	253-256	+20	16, 23, 28, 38, 45, 41
3	C ₂₁ H ₃₀ O ₆	Allopregnan-3(β), 17(β), 21-triol-11, 20-dione	230-238	+66	16, 23, 38, 41
4	C ₂₁ H ₃₀ O ₆	4-Pregnen-11, 17(β), 20, 21-tetrol-3-one	126-129 ^c	+87	23, 38
5	C ₂₁ H ₃₀ O ₆	4-Pregnen-11, 17(β), 21-triol-3, 20-dione	217-220 ^d	+167	16, 18, 38
6	C ₂₁ H ₃₀ O ₆	4-Pregnen-17(β), 21-diol-3, 11, 20-trione	215	+200	16, 28, 29, 32, 38
7	C ₂₁ H ₃₀ O ₆	Allopregnan-3(β), 17(β), 20(β), 21-tetrol	198-200	-1	46, 47
8	C ₂₁ H ₃₀ O ₆	Allopregnan-3(β), 11, 21-triol-20-one	202-204		33
9	C ₂₁ H ₃₀ O ₆	Allopregnan-3(β), 17(β), 21-triol-20-one	230-239	+48	46, 48, 49, 50
10	C ₂₁ H ₃₀ O ₆	Allopregnan-3(β), 21-diol-11, 20-dione	189-191	+94	36, 46, 48, 51
11	C ₂₁ H ₃₀ O ₆	4-Pregnen-11, 21-diol-3, 20-dione	180-182	+223	18, 32, 28, 36, 52, 53, 54
12	C ₂₁ H ₃₀ O ₆	4-Pregnen-17(β), 21-diol-3, 20-dione	207-209		32, 39, 47
13	C ₂₁ H ₃₀ O ₆	4-Pregnen-20, 21-diol-3, 11-dione			34
14	C ₂₁ H ₃₀ O ₆	4-Pregnen-21-ol-3, 11, 20-trione	177-180		28, 33, 36
15	C ₂₁ H ₃₀ O ₆	Allopregnan-3(β), 17(β), 20(α)-triol	222-223	-13 ^e	46, 47
16	C ₂₁ H ₃₀ O ₆	Allopregnan-3(β), 17(β), 20(β)-triol	217-218	-8	32, 46, 47, 55
17	C ₂₁ H ₃₀ O ₆	Allopregnan-3(β), 17(β), 21-diol-20-one	264-266	+31	29, 56, 57
18	C ₂₁ H ₃₀ O ₆	4-Pregnen-17(β)-ol-3, 20-dione	212-215 ^a	+102 ^e	42
19	C ₁₉ H ₂₆ O ₅	4-Pregnen-21-ol-3, 20-dione	141-142	+178	1, 33
20	C ₁₉ H ₂₆ O ₅	11-Hydroxyisoandrosterone	235-238	+85	33, 41
21	C ₁₉ H ₂₆ O ₅	4-Androsten-3, 11, 17-trione	220-223	+262	23, 40, 37

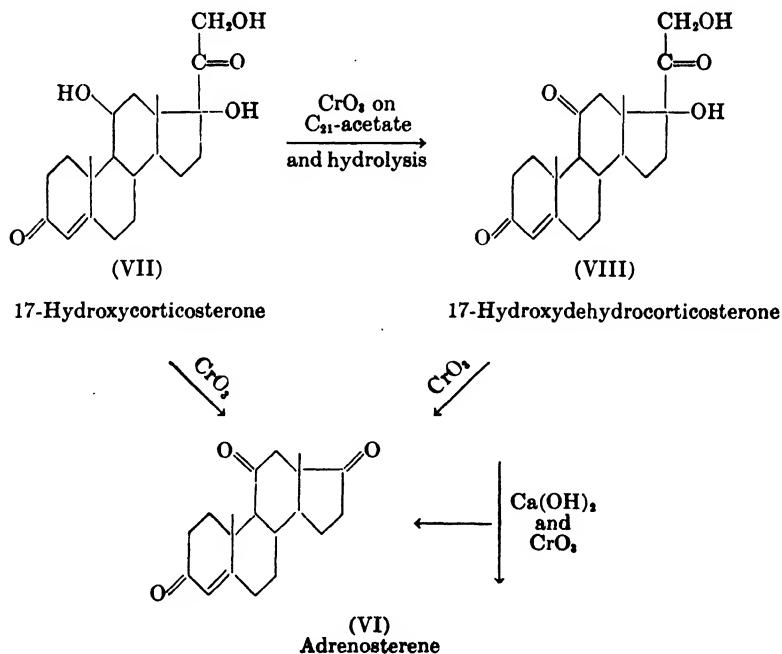
^a Progesterone, oestrone and allopregnan-3(β)-ol-20-one are not included. References (43) and (44). ^b In those compounds bearing a hydroxyl group on carbon atom 17 the configuration is given as 17(β). This steric relationship has been demonstrated only for a number of those compounds without oxygen on C₁₁. References (47, 48, 49, 50, 57, 58, 59). The position of a hydroxyl or ketone group on C₁₁ is generally accepted but not yet proved. ^c The highest corrected melting point given in the literature is recorded. ^d The citation refers to the entries in the bibliography at the end of the article. ^e Characterized only as the diacetate, m. p. 212-213°. ^f In methanol. ^g Uncorrected. ^h In chloroform. ⁱ Monohydrate.

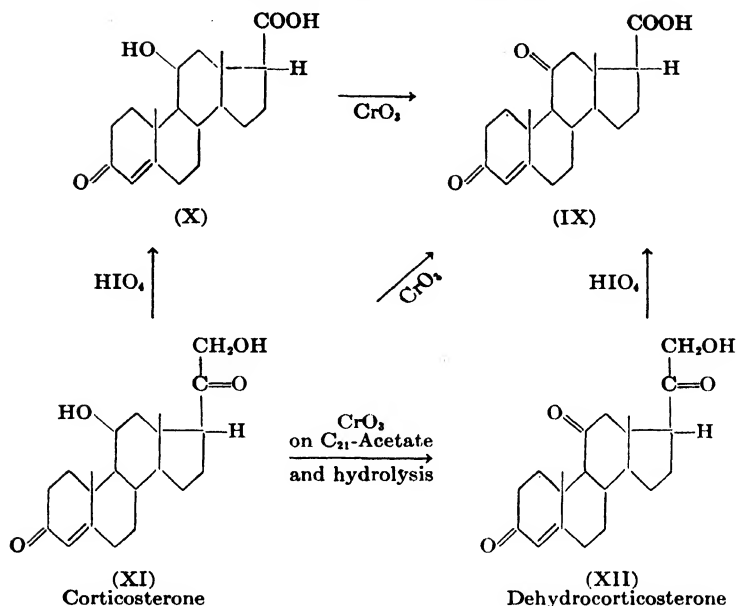
androgenic activity of the compound, $C_{19}H_{24}O_3$ (40). This substance, adrenosterone (IV) reacted as a diketone and was readily reduced with one mol of hydrogen to the saturated compound III. The compound III which likewise reacted as a diketone was also obtained by chromic acid oxidation of the pentol I (41). The results of lead tetraacetate oxidation of I indicated a glycerol grouping with a hydroxyl group in a tertiary position.



Oxidation with either lead tetraacetate or periodic acid gave rise to the monoketone II which was readily converted to either III or V and thence to androstane VI (60). Among other transformation products Reichstein (37) obtained the known androstan-17-one, thereby fixing the position of one of the oxygen atoms on the nucleus, and a new androstanone in which the keto group was unreactive. By assuming the presence of a β -hydroxyl group on C₃ because of the digitonin precipitability of I, II and V, the unreactive keto group could be assigned to C₁₁ or C₁₂ by the system of eliminative reasoning. The position of the single keto group in II fixed the attachment of the 2 carbon side chain (16, 37, 41, 60). Shoppee (61) has recently offered direct evidence for the presence of a hydroxyl group on C₃ by converting the pentol I to androstane-3,17-diol.

The close relationship of 17-hydroxycorticosterone (VII) to 17-hydroxydehydrocorticosterone (VIII) was demonstrated by Reichstein (38). He converted VII to VIII by mild chromic acid oxidation of the mono-acetate and subsequent hydrolysis. Both VII and VIII reacted as diketones and on chromic acid oxidation yielded adrenosterone (VI) (16, 38). The C₂₁O₅ hormones (VII and VIII) were linked directly to corticosterone (XI) and





dehydrocorticosterone (XII), the C_{21}O_4 hormones, by Mason (62) with the conversion of 17-hydroxydehydrocorticosterone (VII) to IX. This same acid had been obtained earlier both by periodic acid oxidation of dehydrocorticosterone (XII) and chromic acid oxidation of corticosterone (XI) (18, 36). The compounds XI and XII react as diketones. The function of the fourth oxygen in the C_{21}O_4 hormones was demonstrated (18, 35) by periodic acid oxidation of corticosterone (XI) to X which with chromic acid was converted to IX. The position 12 was eliminated as a possibility for allocation of the inert oxygen atom by direct comparison of IX with 3,12-diketo-4-etiocolonic acid (63). Direct proof of the carbon skeleton of corticosterone (XI) was its conversion to allopregnane by Steiger and Reichstein (52). More recently Shoppee and Reichstein (54) have converted corticosterone (XI) to a mixture of pregnan-3,20-dione and allopregnan-3,20-dione offering further proof of the presence of the ketonic groups on C_3 and C_{20} and a double bond in position 4-5. Positive proof for the position of the hydroxyl or carbonyl group assigned to C_{11} is still lacking. In a series of studies Reichstein (47) has shown that a number of the C_{17} hydroxylated adrenal steroids, in fact every one he has thus far examined, have

the same relative configuration at C₁₇. He arbitrarily called the configuration which occurs in the natural compounds the β -type (48, 49). While no direct information is available on the configuration of the C₁₇ hydroxyl group in 17-hydroxycorticosterone (VII) or 17-hydroxydehydrocorticosterone (VIII), they are assumed by analogy to be of the β -series. The steric configuration of the C₁₁ hydroxyl in corticosterone and 17-hydroxycorticosterone is unknown but it is assumed to have the same relative position in both compounds.*

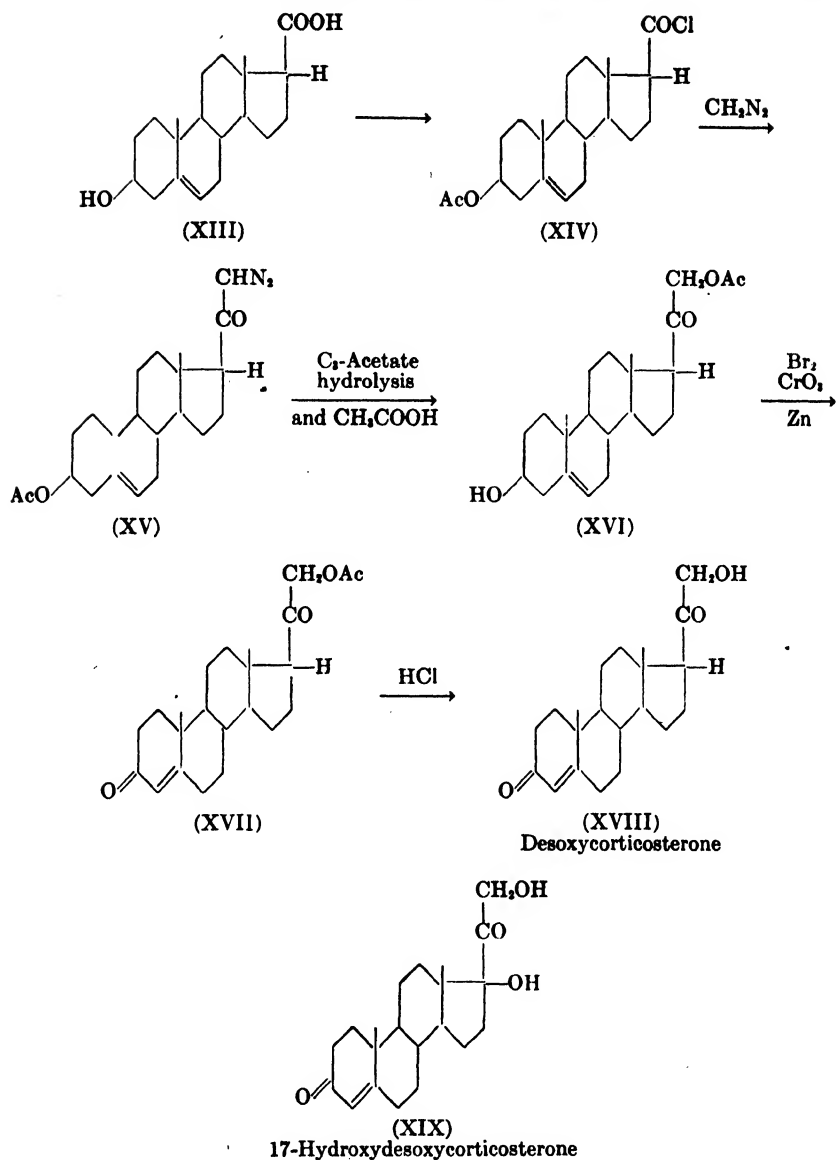
V. Partial Synthesis

Shortly after the available evidence indicated that the then known cortex hormones had three of their four or five oxygen atoms disposed as in 4-pregnen-21-ol-3,20-dione (XVIII) Steiger and Reichstein (1) reported the preparation of this compound from 3-hydroxy-5-etiocholenic acid (XIII) by the series of reactions indicated below. The over-all yield of 4-pregnen-21-ol-3, 20-dione, which they called desoxycorticosterone, was about 10 per cent (20 per cent yield of the 21-acetate). Reichstein and von Euw (64) improved the synthesis by taking advantage of the exceptional stability of the diazoketone (XV). The Oppenauer reaction on the corresponding 3-alcohol yielded the Δ^4 -3-ketone which gave desoxycorticosterone (XVIII) on treatment with mineral acid.

Ehrhart, Ruschig and Aumüller (65) later prepared desoxycorticosterone by the direct oxidation of the 21-methyl group in 5-pregnen-3-ol-20-one or in progesterone with lead tetraacetate. This approach gave products very difficult to purify with a yield in the case of progesterone of less than 3 per cent of the 21-acetate (66). The direct *in vitro* conversion of progesterone to 21-hydroxyprogesterone is of considerable biochemical interest, however, because of the cortin activity of progesterone itself. Another method of directly hydroxylating the 21-methyl group consists in treating the C₂₀-keto compound with potassium persulfate in acetic acid containing a small amount of sulfuric acid (67).

Desoxycorticosterone was prepared from the readily accessible dehydroisoandrosterone by Serini, Logemann and Hildebrand (68). They added

* 17-Hydroxyprogesterone has recently been isolated also in Reichstein's laboratory and its β -configuration proved by partial synthesis of the compound [J. von Euw and T. Reichstein, *Helv. Chim. Acta*, **24**, 879 (1941); D. A. Prins and T. Reichstein, *Ibid.*, **24**, 945 (1941)]. These studies in conjunction with other recent results [T. Reichstein *et al.*, *Helv. Chim. Acta*, **24**, 396, 401, 418, 804, 828 (1941)] make available by partial synthetic methods all seven of the known 17-hydroxylated adrenal steroids having no C₁₁ oxygen, and they likewise complete the direct proof of their C₁₇- β -configuration.



acetylene to dehydroisoandrosterone and reduced the resultant 17-ethynyl-androsten-3,17-diol to the corresponding ethenyl derivative. The β - γ

unsaturated C_3 -OH was converted in the usual manner to the α - β unsaturated C_3 -ketone group by oxidation. The ketone yielded a 17,20,21-triol on treatment with osmic acid which was dehydrated to desoxycorticosterone on distillation over zinc dust.

17-Hydroxydesoxycorticosterone was prepared by Reichstein and von Euw (39) by rearrangement of the 17,20-diol-21-al (69) in boiling pyridine.

VI. Methods of Assay and Comparative Activities of the Hormones

During the past ten years a large variety of assay procedures have been proposed for the evaluation of cortin activity but relatively few of these have been reduced to practice. The few which have been adopted for more or less systematic study fall into two classes, (1) those in which the underlying principle is the maintenance of the adrenalectomized dog or rat, and (2) those in which the efficiency of the muscle of the adrenalectomized rat is measured. Methods of the first class stem from the classical test procedure, the survival of the adrenalectomized animal for a prolonged period of time, while those of the second class have their origin in one of the cardinal symptoms of the Addisonian syndrome, namely, profound muscular weakness. We will discuss only those procedures which have shed some light on the relative activity of the individual hormones.

1. *The Adrenalectomized Dog*

The dog method of assay (70) consists essentially in the determination of the minimum amount of a preparation necessary to maintain the adrenalectomized animal in a normal state. This is accomplished by placing the animals fully recovered from the operation on a maintenance dose and reducing the dose level at 7- to 10-day intervals until the animal exhibits an appr. 100 per cent rise in the blood urea level and also begins to fail clinically. The animals are then reconditioned for further testing by the injection of excess of cortex extract. The method was originally developed as a research tool to study the concentration of cortin activity and for this purpose it served admirably. It is, however, expensive, time consuming and full of pitfalls. On the other hand, it is the most trustworthy method available for the positive demonstration of cortin activity, particularly if the assay period extends over a period of several weeks. The method has been frequently criticized because of the variation in requirements of individual dogs, the nature of the test precluding the use of large numbers of animals on any individual sample. This shortcoming is one common to most bio-

assay procedures. It can be overcome by checking the test animals against a reference standard. In the conduct of this assay it is essential to rigidly standardize the maintenance conditions, particularly the sodium and potassium content of the diet and also the environmental temperature (27, 70). A unit is the minimum daily kilogram dose necessary for maintenance. Using this method Mason, Hoehn, McKenzie and Kendall (36) reported that a dog weighing 13.6 kg. was maintained with 1.5 mg. of corticosterone daily but developed typical symptoms of adrenal insufficiency when the dose was reduced to 1.0 mg. The minimum daily kilogram dose therefore for this particular dog was in the neighborhood of 0.1 mg. 17-Hydroxydehydrocorticosterone was tested by Kendall (27) in one dog (weight unspecified). The animal was maintained for 5 days with 7.5 mg. daily and then for 4 days with 10 mg. daily. On withdrawing treatment the animal failed promptly. Wintersteiner and Piffner (29) found 17-hydroxydehydrocorticosterone to be inactive in one dog at 0.1 mg. per kilo body weight per day, and in two dogs at 0.05 mg. Desoxycorticosterone was reported by Kendall to be six (5-7) times as active as corticosterone on two dogs (33, 71) which would place the daily kilogram dose at about 15 to 20 μ g. The amorphous fraction of Piffner, Wintersteiner and Vars (22, 45) was reported active at a dose level of 2.5 μ g. per kilo of body weight per day in a series of three dogs. More recently Kendall (11, 71, 72) found that the amorphous fraction is active at a dose level of 1 to 2 μ g. per kilo of body weight per day (number of animals not specified).

No data are available on the activity of dehydrocorticosterone or 17-hydroxycorticosterone but it is reasonable to expect that the potency of these two compounds in the dog assay is comparable to that of corticosterone and 17-hydroxydehydrocorticosterone, respectively. It is obvious that more extensive observations are needed on the activity of the several cortex hormones by the dog method, particularly since this method has recently been recognized by the Council on Pharmacy and Chemistry of the American Medical Association (73) for the estimation of cortin activity in gland extracts.*

A number of observations have been made on the activity of the cortex hormones in the adrenalectomized dog under conditions differing from those employed in the dog assay. In a brief note corticosterone was reported to

* In a series of seven dogs the daily average minimal maintenance dose of desoxycorticosterone acetate when administered in oil was established at 0.286 mg. per day on a "2% NaCl diet" and 1.56 mg. in a series of four dogs on a "1% NaCl diet." A two-week test period was used (Cleghorn, R. A., Fowler, J. L. A., Wenzel, J. S., and Clarke, A. P. W., *Endocrinology*, 29, 535 (1941)). These dosage levels are roughly equivalent to 0.03 and 0.16 mg. per kilogram of body weight per day but these authors emphasize the lack of correlation between the size of the minimal maintenance dose and the weight of the test animal.

be active in a series of four animals in both curative and preventive experiments (19), 0.25 to 0.5 mg. of corticosterone being equivalent in potency to the extract from 50 gm. of glands. Since 50 gm. of glands ordinarily yield 50 to 100 dog units (74) corticosterone on the basis of these experiments might be considered to have a potency of 100 to 400 dog units per mg. Such an inference is not in harmony with the findings outlined above. Thorn, Engel and Eisenberg (75) made a careful study of the effect of desoxycorticosterone on the sodium, chloride and potassium balance in one adrenalectomized dog on an average diet. One mg. of desoxycorticosterone daily maintained the electrolyte balance in a normal state but a minimal level was not established. In a series of five animals on a diet low in sodium, chloride and potassium they found (76) that 1 mg. of desoxycorticosterone was equivalent to the extract from *ca.* 280 gm. of glands. Such an amount of gland substance should yield *ca.* 280 to 560 units of activity by the usual dog method of assay. Desoxycorticosterone therefore appears to be more active in short term balance experiments than it does in the dog assay method itself. In this connection, however, it should be pointed out that the serum sodium is usually significantly depressed before a rise of 100 per cent is observed in the blood urea or non-protein nitrogen, especially if the assay extends over a period of several weeks (22). Unfortunately no comparative data on balance experiments in the adrenalectomized dog are available for the amorphous fraction.

If one accepts the available dog assay data at face value and assumes a potency of 5 and 30 dog units per mg., respectively, for 17-hydroxycorticosterone and 17-hydroxydesoxycorticosterone then a balance sheet for 1000 pounds of glands can be drawn up as follows:

	Dog units
Expected yield, 1 dog unit per gm. of gland	450,000
Corticosterone, 350 mg. at 10 dog units per mg.	3500
Dehydrocorticosterone, 350 mg. at 10 dog units per mg.	3500
17-Hydroxycorticosterone, 40 mg. at (5?) dog units per mg.	200
17-Hydroxydehydrocorticosterone, 500 mg. at 5 dog units per mg.	2500
Desoxycorticosterone, 15 mg. at 60 dog units per mg.	900
17-Hydroxydesoxycorticosterone, 10 mg. at (30?) dog units per mg.	300
Total dog units in isolated crystalline hormones	10,900
	10,900
Dog units of activity not accounted for as pure crystalline hormones	439,100

If the yields of isolated crystalline hormones are assumed to represent

only 10 per cent of their glandular concentration then over 75 per cent of the physiological activity would yet remain to be accounted for in the non-crystalline residues, the bulk of it in the amorphous fraction. Using the dog method of assay Wells and Kendall (11) estimated that 90 per cent of the activity in adrenal extract was present in the amorphous fraction.

2. *The Immature Adrenalectomized Rat*

Prior to the work of Kutz (77) the rat was not considered suitable for test purposes because a large percentage of animals survived adrenalectomy indefinitely. This worker found that the mortality approached 100 per cent if the animals were adrenalectomized at 28 days of age. The survival and growth of the immature adrenalectomized rat has since been used by a number of investigators as a measure of cortin activity (24, 25, 78, 79, 80, 81). The conditions employed such as numbers of test animals, percentage survival, period of test, extent of growth, diet, *et cetera*, have varied in different laboratories so that the results are not comparable on any unitage basis. Grollman (25) found that a daily dose of 1.0 mg. of desoxycorticosterone acetate permitted test rats to survive and to grow as judged by body weight but the data were not sufficiently extensive to definitely establish this level as a minimum. Corticosterone at a 0.5-mg. level gave results comparable to those obtained with desoxycorticosterone acetate at 0.1 mg. The crude hormone mixture, on the other hand, was estimated to be 10 times as active as desoxycorticosterone acetate. Test groups of six rats each were used.³ Kuizenga and Cartland (30) found that a daily dose of 0.25 mg. of corticosterone was necessary to maintain life and permit growth (1 gm. per rat per day, 5 animals). Dehydrocorticosterone was equally active (5 animals). A dose level of 0.5 mg. of 17-hydroxycorticosterone, and 1.0 mg. of 17-hydroxydehydrocorticosterone produced a comparable effect in groups of 3 rats each. In contrast the amorphous fraction was active at a daily dose level of 30 to 50 μ g. when assayed on groups of 20 rats each. These workers in a later study (82) found desoxycorticosterone acetate active at a dose level of about 30 μ g. using groups of 5 test animals each, an activity comparable to that reported earlier (30) for the amorphous

³ Grollman (25) reports the activity of a crystalline product as 100 times that of desoxycorticosterone and 1000 times that of corticosterone. This substance is not discussed here since it has been characterized only in a very preliminary way. The author gives an empirical formula of $C_{19-20}H_{28-30}O_5$. Its melting point is 182-183°. It is said to have "the character of a poly-hydroxy α - β unsaturated steroid" and to be very unstable. It was reported as being completely inactivated on filtering an alcoholic solution through aluminum oxide.

fraction. No explanation is apparent for the contradictory findings of Grollman (25) and those of Kuizenga and Cartland (80, 82) on the relative activity of desoxycorticosterone and the amorphous fraction. Waterman (81) in a brief note recorded the effects of a single injection of a substance on the survival of immature adrenalectomized rats. The survival was recorded to 0.1 day, the injection given on the second day following adrenalectomy, and conclusions drawn on differences of 1 or 2 days in the average survival of a group of 4 to 10 animals. Corticosterone butyrate was active at 1 mg. while the benzoate was inactive. Desoxycorticosterone acetate at 0.5, 1 and 3 mg. was considered active as was 17-hydroxycorticosterone at 1.5 and 3 mg. but the latter compound was inactive at a 2-mg. level. The amorphous fraction was inactive at 2 mg. The data are not sufficiently extensive to be considered decisive.

9. *Efficiency of Muscle*

Everse and de Fremery (83) devised a method of assay using as a criterion of activity the height of the contractile response of the calf muscle in the leg of the adrenalectomized rat. The detailed description of the method is given by R. W. Spanhoff in Reichstein's monograph (13).

The adult animal four days following adrenalectomy is anesthetized with ether and an ergograph made of the response of the calf muscle to an intermittent tetanizing current. The animal is then injected twice daily for four days and a second ergograph obtained. If the ergographs from 4 of 6 test animals are comparable to that from a normal rat the injected substance is considered to be active. A unit is one daily dose.

Reichstein (18) reported the activity of corticosterone as one unit in 0.8 mg., while 17-hydroxycorticosterone was only about one-half as active (1 unit in 1.5 mg.) (13). The amorphous fraction had 1 unit in *ca.* 0.3 mg., an activity two or three times that of corticosterone. Desoxycorticosterone was later reported to be ten times as active as corticosterone which would make it about 3 to 5 times as active as the amorphous fraction (33). No data are recorded for dehydrocorticosterone by this method of assay. 17-Hydroxydehydrocorticosterone was inactive at 0.8 mg. (13).

In the method of assay developed by Ingle (84) the criterion of activity is the work capacity of the gastrocnemius muscle.

The adult rat is adrenalectomized under sodium phenobarbital anesthesia and usually within an hour the gastrocnemius is fixed and loaded. It is stimulated with an intermittent direct current and the work accomplished is totaled on automatic work recorders. Heron, Hales and Ingle (85) described the technique and apparatus in detail. Matched groups of 3 to 5 male rats usually were employed and the compounds injected subcutaneously at 6-hour intervals. No unit was defined. Corticosterone, dehydrocorticosterone,

17-hydroxycorticosterone and 17-hydroxydehydrocorticosterone were about equally active. A dose of *ca.* 0.5 mg. every 6 hours allowed nearly a normal work performance. Desoxycorticosterone or its acetate in doses up to 3 mg. had little if any effect on the work output (86).

In a later paper (87) these observations were confirmed and it was shown that adrenalectomized rats maintained for 7 days with 17-hydroxydehydrocorticosterone (1 to 2 mg. per day) had a far better work output at the end of the period than did animals treated with desoxycorticosterone or its acetate at higher dosage levels. The amorphous fraction was about one-third to one-half as active as 17-hydroxydehydrocorticosterone but much more active than desoxycorticosterone (88).

The foregoing assay results have been reviewed in some detail to impress the reader with the limited data thus far assembled. The generalities which may be drawn from the observations are summarized in Table II.

The only direct experiments on the interrelationship of the various units of activity are those of Cartland and Kuizenga (80) who compared the rat and dog methods. They found that 1 rat unit was equivalent to 22 dog units. It should be emphasized that this equivalence was shown to exist only for a crude adrenal extract. On the basis of yield of activity per unit weight of gland tissue, Reichstein (13) estimated that one Everse-de Fremery unit in the crude extract was equivalent to 50-100 dog units. The results by the Everse-de Fremery test do not entirely parallel the results on life maintenance (see Table II). Ingle (84) at first reported good agreement between assay results by his method and those by the dog method using less pure preparations. His later work on the individual hormones (87) has demonstrated the accidental nature of this relationship.

TABLE II
COMPARISON OF ACTIVITY OF THE CORTICAL HORMONES BY DIFFERENT METHODS OF ASSAY
(Numbers indicate the order of activity)

	Maintenance of life			Muscular efficiency	
	Dog	Rat		Tetanic contraction ^c	Work output ^d
		(a) ^a	(b) ^b		
Corticosterone	3	3	2	3	1
17-Hydroxycorticosterone	4	..	3	..	1
Dehydrocorticosterone	3	..	2	..	1
17-Hydroxydehydrocorticosterone	4	..	3	4	1
Desoxycorticosterone	2	2	1	1	3
Amorphous fraction	1	1	1	2	2

^a Grollman. ^b Cartland and Kuizenga. ^c Everse-de Fremery. ^d Ingle.

VII. Comparative Effects on Carbohydrate Metabolism

During the past two or three years evidence has accumulated to show that the cortex hormones vary significantly in their effects on carbohydrate metabolism. The effect of adrenal cortex extract on the deposition of liver glycogen in the rat both normal and adrenalectomized, was early demonstrated by Britton and Silvette (89). Long and his colleagues (90) in the course of their studies on the relation of the adrenal cortex and anterior pituitary to protein and carbohydrate metabolism observed the diabetogenic action of cortex extract in the partially depancreatized rat. These workers found that corticosterone, dehydrocorticosterone and 17-hydroxydehydrocorticosterone markedly stimulate the glycosuria in the partially depancreatized rat. Desoxycorticosterone, on the other hand, has little or no effect when administered in comparable dosage. Wells (91) compared corticosterone, 17-hydroxydehydrocorticosterone, desoxycorticosterone and the amorphous fraction in the fasting phlorizinized adrenalectomized rat. Corticosterone and 17-hydroxycorticosterone restore gluconeogenesis (as measured by urinary glucose and nitrogen excretion) to an essentially normal level. Desoxycorticosterone and the amorphous fraction in contrast have much less effect. The small number of test animals preclude any quantitative conclusions. Thorn and his associates (92) also observed that cortex extract, corticosterone or 17-hydroxydehydrocorticosterone cause the adrenalectomized rat to respond as a normal animal to phlorizin poisoning. Desoxycorticosterone is ineffective. These findings are in agreement with those of Wells (91). Thorn (93) extended his observations to patients suffering with Addison's disease who also exhibited symptoms of a disturbed carbohydrate metabolism, *e. g.*, a low fasting blood sugar, a tendency to develop marked hypoglycemia, an abnormal glucose tolerance curve, *et cetera*. Desoxycorticosterone failed to correct these abnormalities although they were corrected with either cortex extract, corticosterone or 17-hydroxydehydrocorticosterone. Grattan and Jensen (94) compared the cortex hormones for their anti-insulin effect in mice and also for their influence on the deposition of liver glycogen. In conformity with the results enumerated above they found that corticosterone, 17-hydroxycorticosterone and 17-hydroxydehydrocorticosterone were very active in both respects. Desoxycorticosterone was essentially inactive. It should be noted that the hormones influence carbohydrate metabolism in essentially the same order as their effectiveness in the Ingle work test (86) (see Table II). The oxygen function on C₁₁ is apparently a definite structural requirement for diabetogenic activity. The contributory effect, if any, of

the tertiary hydroxyl group to the diabetogenic effect in 17-hydroxycorticosterone and its 11-dehydro analogue is not established.*

VIII. Other Biological Reactions

The possibility that desoxycorticosterone is an important constituent of the amorphous fraction is not eliminated by the results of bioassay or by the studies on carbohydrate metabolism. Observations which have been made on overdosage are of special significance in this respect. Loeb, Atchley and their co-workers (95) first noted the effects of overdosage in patients with Addison's disease. Desoxycorticosterone not only caused the desired retention of sodium and water but in the dosage employed, hypoproteinemia, marked edema and cardiac insufficiency developed. These results prompted them to study the effect of the compound on normal dogs (96, 97). In a series of 4 animals a dose of 20 to 25 mg. per day resulted in the development of a peculiar type of paralysis. The most striking finding was a lowered serum potassium level, the potassium falling from an average normal of 4.4 to 2.4 m. eq. per liter. The serum sodium was slightly elevated. On continued treatment the animals exhibited polyuria and polydipsia. In terms of dog units of activity such a dosage is equivalent to the daily administration of the extract from about 1000-2000 gm. of glands. It is a well-known fact that such a quantity of extract produces no overdosage effects in dogs nor in the writer's experience does the amorphous fraction in equivalent amount. This is evidence in support of the view that the high cortin activity of the amorphous fraction as demonstrated in both the rat and the dog is not due to its content of desoxycorticosterone. That the rat is a peculiar animal in so far as its adrenal physiology is concerned is again attested by the fact that in this form desoxycorticosterone produces no comparable symptoms of overdosage (98). However, Wells and Kendall (11) found that in rats desoxycorticosterone caused a depression in the serum potassium level which was not the case with either corticosterone or the amorphous fraction. They also attempted to differentiate the cortex hormones on the basis of their ability to cause adrenal and thymus atrophy. Their findings that desoxycorticosterone differed qualitatively from the other cortex hormones in failing to produce adrenal or thymus atrophy in the rat have not been substantiated in later experiments. Selye (99) and Carnes *et al.* (98) observed adrenal atrophy following desoxycorticosterone

* Ingle has informed the author in a private communication that 17-hydroxydesoxycorticosterone is non-diabetogenic in the partially depancreatized rat at twice the active dose level of 17-hydroxydehydrocorticosterone.

injections in the rat while Ingle (100) noted that desoxycorticosterone produced thymus atrophy. Apparently these atrophic effects are entirely quantitative rather than qualitative.

Since the sodium and chloride retaining effect of cortex extracts in normal dogs had been well established (101, 102), Thorn, Engel and Eisenberg (75) compared corticosterone, dehydrocorticosterone, 17-hydroxycorticosterone and desoxycorticosterone. They found that, in general, the sodium and chloride retention paralleled the cortin activity as measured in the adrenalectomized dog, desoxycorticosterone being active at 0.5-1 mg., corticosterone or dehydrocorticosterone at 4 mg., while 17-hydroxycorticosterone was inactive at 8 mg.⁵ All of these compounds caused a potassium diuresis which, however, is quite non-specific. A potassium diuresis followed the injection of adrenalin as well as some of the adrenal steroids known to have no cortin activity. In the rat, as G. B. West (103) has shown, almost any irritating injection will cause a potassium diuresis. The sodium and chloride retention is likewise non-specific for the cortex hormones. Thorn and Harrop (104) and Thorn and Engel (105) demonstrated the sodium and chloride retaining effect of progesterone, estrone, α -estradiol and testosterone in both the adrenalectomized and the normal dog. Of the sex hormones progesterone is the only one having some cortin activity (106) and that is of a relatively low order. There is some evidence, however, that a substance can have cortin activity but be without effect in the normal dog on the sodium and water retention. Hartman and Spoor (10) have recently reported the preparation of a cortex fraction as active as desoxycorticosterone in maintaining the life of the adrenalectomized cat but having no effect on sodium retention in the normal dog. These important and rather unexpected findings await confirmation.

Desoxycorticosterone on subcutaneous injection is about one-sixth to one-tenth as active as progesterone on the endometrium of the rabbit (107, 108, 109). This offers a clean-cut means of differentiating this compound from at least some of the other crystalline cortex hormones as well as from the amorphous fraction. Adrenal extract and 17-hydroxydehydrocorticosterone have been shown (110, 30) to be entirely inactive in this respect.

⁵ Thorn, Engel and Lewis (*Science*, 94, 348 (1941)) just recently report that 17-hydroxycorticosterone and 17-hydroxydehydrocorticosterone in doses of 5 to 8 mg. cause an increased sodium and chloride excretion in the normal dog. A dose of 25 mg. of the latter compound also caused an increased sodium and chloride excretion in an adrenalectomized dog while at a 6 mg. dose level it caused a marked increase in the 24-hour excretion of sodium chloride in the normal rat. Control studies in the normal dog demonstrated again the sodium chloride retaining effect of corticosterone at a 4 mg. dose level and desoxycorticosterone at 1 mg.

Of particular significance is the inactivity of the amorphous fraction at a very high dosage level (equivalent to about 50-100 pounds of glands). No observations have been made on corticosterone, dehydrocorticosterone or 17-hydroxycorticosterone. Desoxycorticosterone has been reported to have androgenic effects (111), but these findings have not been confirmed (112, 113).

In this article the possibility of an unknown activator of one of the known hormones was not considered in attempting to account for the cortin activity of the amorphous fraction. Neither was the question of the synergistic action of two or more of the known hormones discussed. The reason for this is that no conclusive data on the latter point are available and an activator if present may be classed as a hormone in the broad sense of the term.

IX. Activity of Related Steroids

The simplest structure consonant with cortin activity is exemplified by desoxycorticosterone, a 4-pregnene skeleton with a keto group on C₃ and a ketol side chain. A large number of related steroids have been tested for cortin activity by different workers. The only generally accepted exception to these minimal structural requirements is progesterone. The writer has not included it as a cortex hormone because it is not a constituent so far as is known of the crude cortin-active hormone mixture. It was isolated (43) from a cortin-inactive fatty fraction of the gland which was separated early in the process. The life maintenance activity of the corpus luteum (progesterone) was suspected since the early demonstration by Stewart and Rogoff (2) that the pregnant or pseudo-pregnant bitch survived adrenalectomy for the respective period. In recent years progesterone has been shown to have cortin activity in the ferret (106), rat (114, 115, 116) and mouse (117). Its action on the sodium and chloride balance of the adrenalectomized and normal dog is about one-fourth as great as that of corticosterone (105). A single large dose (30 mg.) had no effect on the sodium diuresis in a case of Addison's disease (95). It is inactive in the work test (118) and is without diabetogenic or anti-insulin activity in the mouse (94). It does not influence the liver glycogen level in the adrenalectomized rat (119). The failure of progesterone to influence carbohydrate metabolism would therefore suggest its classification with desoxycorticosterone.

Selye (120) has just recently reported in a preliminary note that 3-hydroxy-21-acetoxy-5-pregnen-20-one is almost as active as desoxycorticosterone acetate in maintaining life and permitting growth in young adrenalectomized rats. These results are very unexpected since Reichstein

(13) found this compound, which is an intermediate in the synthesis of desoxycorticosterone, entirely inactive in the Everse-de Fremery test at a dosage level 50 times that at which desoxycorticosterone acetate is active. Ingle reported this compound inactive in his work test (118). Selye also found 3-hydroxy-5-pregnen-20-one active in the life maintenance of the rat, as judged by the blood picture (hemoglobin, sugar, non-protein nitrogen levels) in a short (4-day) test period. The results of survival experiments using a longer test period will be awaited with interest.

The specificity of the C_{17} - α configuration of the two carbon side chain for cortin activity was demonstrated by Shoppee (121) who prepared 17-iso-desoxycorticosterone. This is the C_{17} epimer of desoxycorticosterone. It was inactive in maintaining the young adrenalectomized rat at a level three times as great as the necessary dose level of desoxycorticosterone.

Reich and Reichstein (122) prepared 4-pregnen-3,20-dione-21-al with the thought that a keto or hydroxy aldehyde of the pregnene series might be a factor in the high cortin activity of the amorphous fraction. It was found to be less active than corticosterone in the Everse-de Fremery test; 1 mg. gave a negative while 2.5 mg. gave a positive response. No observations have been reported on the life maintenance activity in adrenalectomized animals. Reichstein and his associates have more recently prepared 4-pregnen-17,20-diol-3-one-21-al-20-acetate (69) and 4-pregnen-20-ol-3-one-21-al (123) but no results on the biological tests have been published.

X. Metabolism of the Adrenal Steroids

The occurrence of a large number of closely related steroids in the adrenal cortex has added many problems to the study of intermediary steroid hormone metabolism. The three structural features which render the cortex hormones of particular interest are the hydroxyl or carbonyl oxygen on C_{11} , the tertiary hydroxyl on C_{17} , and the ketol group. To date no observations have been made by the classical metabolic procedure of introducing the compound into the organism and studying the excretory products. The feasibility of such work has been limited by the available supplies of the active compounds other than desoxycorticosterone. Current views on metabolism rest largely on the effort to interpret the results of isolation and structural studies of the urinary steroids on the basis of structural correlation with the hormones. Such efforts are admittedly almost purely speculative.

That the cortex hormones are rapidly inactivated in the animal body is indicated, first, by the fact that the effects of an injection in an adrenalect-

tomized animal wear off rapidly and, second, injected hormones are not excreted in the urine, at least by the normal dog (74). Inactivation of the hormones by reduction is an attractive hypothesis especially since numerous reduction products of the cortex hormones isolated from the gland have been demonstrated to be physiologically inactive. Cortin activity is lost, for example, by reduction of the C_3 or C_{20} carbonyl group to hydroxyl or saturation of the 4:5 ethylenic bond. That such reduction may be one mechanism of inactivation in the body is rendered probable by the results of Callow (124), Dorfman and others (125) on the conversion of testosterone in man to androsterone and etiocholan-3(α)-ol-17-one, and by the demonstration of Venning and Browne (126) and Buxton and Westphal (127) of the conversion of progesterone to pregnandiol. Marker (128) has indicated the various pathways by which a number of the cortin-inactive steroids isolated from the gland (see Table I) might be derived by reduction of the cortin-active compounds. It remains for future work to demonstrate the occurrence of such reactions in the body. The isolation from adrenal extracts of both allopregnan-3(β),17(β),20(β)-triol and its C_{20} epimer is certainly strong evidence for the reduction of the C_{20} carbonyl group. While the occurrence in glandular extracts of unsaturated alcohols of the cholesterol type can be expected according to Marker, none has as yet been isolated. If inactivation of the hormones by reduction is the actual mechanism involved then the presence of the many reduction products in the gland indicates that a certain amount of inactivation takes place prior to discharge of the hormones from the gland—a rather wasteful procedure. A comparable situation exists in the corpus luteum in which allopregnan-3(β)-ol-20-one occurs along with progesterone.

The progestational action of parenterally administered desoxycorticosterone raises the question of whether the compound is progestationally active as such or whether it is first converted in the body to progesterone. McGinty (129) has found that desoxycorticosterone is inactive when applied locally to the endometrium of the sensitized rabbit (inactive at 500 μ g., progesterone active at 1 μ g.). Such local inactivity suggests that one pathway for the metabolic degradation of desoxycorticosterone may be the removal of the primary hydroxyl group. An increased excretion of pregnandiol glucuronide following the administration of desoxycorticosterone to normal men (130) is also in favor of the view that desoxycorticosterone is converted to progesterone. In this connection it is interesting to note that no C_{21} -hydroxylated steroid has as yet been isolated from urine but this may be due to the vigorous hydrolytic procedures employed in the preparation of the concentrates.

The recognition of excessive urinary excretion of estrogens (131) and androgens (132) by patients suffering from adrenal cortical tumors was soon followed by chemical examination of the steroid fractions prepared from the urine of such patients. These studies have led to the isolation of four new compounds, which do not occur in, or at least have not thus far been isolated from, normal urine.

Burrows *et al.* (133) examined the urine of a man suffering from adrenal feminism. He excreted large amounts of estrogens as measured biologically. The estrogens were not identified chemically but it was suspected that the chief estrogen excreted was estrone. These workers isolated a compound, $C_{19}H_{26}O$, and demonstrated its structure to be 3,5-androstadien-17-one. The rather drastic acid hydrolysis of the urine employed in the isolation made these workers feel that the compound was an artifact and not present in the urine as such. Later workers (134) isolated this substance from the urine of a girl with adrenal virilism using somewhat similar methods and suggested 4-dehydro-androsterone or 4-dehydroisoandrosterone as possible urinary precursors but no C_3 alcohol unsaturated in the 4-5-position is known to occur in the animal body or its excretion products.

The two isomeric triols, $C_{21}H_{36}O_3$, isolated by Butler and Marrian (135, 136) from the urine of adult female patients with adrenal tumors are of interest. The structure of one was demonstrated to be pregnan-3(α),17,20-triol while the tentative structure, pregnan-3(β),17,20-triol was favored for the other. These are the only steroids known to occur in urine which are hydroxylated in the 17-position. The fact that none has been isolated from normal urine may be due not to their absence but to their transformation by the strong acid hydrolysis which most workers have employed in preparing urine concentrates.

Wolfe, Fieser and Friedgood (134) have isolated a new androsten-3-ol-17-one from the urine of a girl with adrenal virilism. From their experimental findings the double bond could occupy the position 6,7-, 7,8-, 9,11- or 11,12-. These workers favored an 11,12-unsaturation since Shoppee and Reichstein (51) had found that the 11-hydroxylated adrenal steroids were readily dehydrated with hydrochloric acid giving rise to an unsaturation presumably at the 11,12-position. No steroids of the androstane, pregnane or allo-pregnane series with an oxygen function on C_{11} are known to occur in either normal or pathological urine. The only C_{11} -hydroxylated steroids isolated from urine are uran-3(α),11,20-triol, uran-3(β),11-diol, and uran-11-ol-3-one which are epimeric with the corresponding pregnane derivatives at C_9 (137). They were obtained from a concentrate prepared from acid hydrolyzed urine of pregnant mares. The 11-hydroxyl group in the urane series

is not hindered and apparently is not readily removed by dehydration. Marker (128) has discussed the possibility of the formation of the urane type of compound by reduction of the enolized 11-keto adrenal steroids. Androsterone (134), dehydroisoandrosterone (134, 138), isoandrosterone (136), etiocholan-3(α)-ol-17-one (136, 134) and pregnandiol-3(α),20(α) (135) have been isolated from the urine of women with adrenal cortical tumors (virilism). These steroids, however, with the exception of isoandrosterone also occur but in much lower concentration in the urine of normal men (124, 127, 138), normal women (139, 140, 141) and ovariectomized women (142).⁶ The occurrence of these compounds in the urine of ovariectomized women together with the isolation of androsterone, dehydroisoandrosterone and etiocholan-3(α)-ol-17-one from the urine of eunuchs is strong evidence as pointed out by Hirschmann (142) and by Callow and Callow (143) for the adrenal origin of at least a part of the urinary 17-keto steroids normally excreted by both normal men and women.

The great ease with which the side chain in the cortex hormones bearing a tertiary hydroxyl group on C₁₇ can be oxidatively or hydrolytically removed to yield the androgen, adrenosterone or its 11-dihydro derivative, prompted Marrian (144), Wintersteiner and others (145) to suggest that in cases of virilism of adrenal origin the excessive excretion of androgens may be caused by a similar reaction. The *in vivo* degradation of 17-hydroxyprogesterone to 4-androsten-3,17-dione would likewise explain its androgenic activity. However, the androgenic activity of 17-hydroxyprogesterone can be readily demonstrated in the castrate rat whereas large doses of 17-hydroxydehydrocorticosterone are entirely without androgenic effect. These observations indicate that 17-hydroxyprogesterone is androgenic *per se* and that the side chain of 17-hydroxydehydrocorticosterone is not readily cleaved in the body. Such cleavage might, of course, be readily effected by tumorous cortical tissue. In fact, only in those cases of virilism involving gross lesions of the cortex are significantly increased amounts of androgens excreted in the urine (146).

The high state of oxidation of some of the cortex hormones has made it increasingly difficult to view the steroid hormones as arising from the metabolic degradation of cholesterol. Reichstein (12, 38) suggested the interesting possibility of the formation of the cortex hormones by the condensation of 3-carbon carbohydrate residues, for example, dihydroxyacetone, or glyceraldehyde, followed by reduction. If a mechanism of this type is involved the animal body can be reasonably expected to yield steroids of

⁶ Pearlman recently announced the isolation of isoandrosterone from the urine of normal women (*J. Biol. Chem.*, 136, 807 (1940)).

even a higher state of oxidation than the cortex hormones. None has thus far been found.

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